SIRT1 activator SRT3025 provides atheroprotection in ApoE$^{-/-}$ mice by increasing hepatic LDL receptor

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1. Summary

1.1 Summary (English version)

Elevated levels of LDL-cholesterol play a crucial role in the development of atherosclerosis. Concordantly, clearance of excess LDL-cholesterol from the blood stream by hepatic LDL receptor (LDL-R) is known to provide atheroprotection. The NAD^+-dependent deacetylase SIRT1 has been described to be protective in lipid dysfunction and to mediate beneficial effects of caloric restriction, while its role in atherosclerosis remains controversial.

To address this controversy we studied the effects of pharmacological SIRT1 activation in two different models of atherosclerosis - apolipoprotein E knockout ($ApoE^{-/-}$) and LDL receptor knockout ($LDL\text{-}R^{-/-}$) mice. $ApoE^{-/-}$ mice fed a high-cholesterol diet (1.25% w/w) and supplemented with the SIRT1 activator, SRT3025 (400 mg/kg/d) for 12 weeks showed less atherosclerosis, and lower plasma levels of total cholesterol and LDL-cholesterol as well as inflammatory cytokines IL-6 and MCP-1. Hepatic LDL-R and PCSK9 expression were increased with a reduction in secreted plasma PCSK9 levels. *In vitro* SRT3025 caused a reduction in PCSK9 secretion with a similar SIRT1-dependent increase in LDL-R expression in AML12 hepatoma cells. In $LDL\text{-}R^{-/-}$ mice, the atheroprotective effects of SRT3025 observed in $ApoE^{-/-}$ mice were abolished whereas the decrease in plasma levels of the inflammatory cytokines IL-6 and MCP-1 was maintained.

Our data provide evidence that SIRT1 activation is atheroprotective by reducing plasma LDL-cholesterol levels through an increase in liver LDL-R expression and reduced PCSK9 secretion. Our findings underline the potential of SIRT1 activation as a therapy against atherosclerosis.

1.2 Summary (German Version)

Erhöhte Plasmaspiegel von LDL-Cholesterin sind ursächlich an der Entstehung der Atherosklerose beteiligt. Eine Reduktion von überschüssigem LDL-Cholesterin aus dem Kreislauf durch den hepatischen LDL-Rezeptor (LDL-R) wirkt hingegen atheroprotektiv. Die NAD^+ - abhängige Deacetylase SIRT1 wirkt protektiv in Lipidstörungen und vermittelt günstige metabolische Effekte der Kalorien-Restriktion. Jedoch kamen verschiedene Studien zu unterschiedlichen Ergebnissen bezüglich der Rolle von SIRT1 in der Arteriosklerose.

In der vorliegenden Arbeit wurde daher in zwei Maus-Modellen der Atherosklerose - Apolipoprotein E-Knockout ($ApoE^{-/-}$) und LDL-Rezeptor-Knockout ($LDL\text{-}R^{-/-}$) - der Effekt einer pharmakologischen SIRT1-Aktivierung untersucht. $ApoE^{-/-}$ Mäuse, welche für 12 Wochen eine cholesterinreiche Diät (1,25% w / w) ergänzt durch den SIRT1 Aktivator SRT3025 (400 mg / kg / d) erhielten, entwickelten weniger Arteriosklerose und geringere Plasmaspiegel an Gesamt- und LDL-Cholesterin, sowie der inflammatorischen Zytokine IL-6 und MCP-1. Die hepatische LDL-R und PCSK9 Protein-Expression war erhöht, die Plasma-Spiegel von PCSK9 dagegen erniedrigt. *In vitro* verursachte SRT3025 eine Verringerung der PCSK9 Sekretion und einen SIRT1-abhängigen Anstieg der LDL-R-Expression in AML12 Zellen. Entsprechende Tierversuche in $LDL\text{-}R^{-/-}$ Mäusen zeigten einen Verlust der atheroprotektiven Wirkung von SRT3025 mit einer Verringerung der entzündlichen Zytokine IL-6 und MCP-1 im Plasma.

Zusammenfassend zeigen unsere Daten, dass SIRT1-Aktivierung atheroprotektiv wirkt, vermittelt durch eine Reduktion von Plasma LDL-Cholesterin durch eine Erhöhung der Leber LDL-R-Expression und die

Reduktion der PCSK9 Sekretion. Unsere Ergebnisse unterstützen das Potential der SIRT1-Aktivierung als potentielle Therapie gegen Arteriosklerose.

2. Abbreviations

AML12	Alpha mouse liver 12
ApoB100	Apolipoprotein B-100
ApoE	Apolipoprotein E
BODIPY FL	Boron-dipyrromethene flourophore
CD38	Cluster of differentiation 38
CD68	Cluster of differentiation 68
CDK5	Cyclin-dependent kinase 5
CHD	Coronary heart disease
CR	Caloric restriction
EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
FH	Familial hypercholesterolemia
FOXO	Forkhead Box O
HDL-cholesterol	High-density lipoprotein cholesterol
HMGCoA	3-hydroxy-3-methylglutaryl Coenzyme A
IL-6	Interleukin 6
LDL	Low-density lipoprotein
LDL-cholesterol	Low-density lipoprotein cholesterol
LDL-R	Low-density lipoprotein receptor
LXR	Liver X receptor
MCP-1	Monocyte chemo-attractant protein 1
mmLDL	minimally modified LDL
oxLDL	oxidized LDL

NA	Nicotinic acid
NAD	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
NMN	Nicotinamide mononucleotide
NAMPT	Nicotinamide phosphoribosyltransferase
NR	Nicotinamide riboside
PARP	Poly (ADP-ribose) polymerase
PCSK9	Proprotein convertase subtilisin/kexin type 9
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
p65	Nuclear Factor Kappa-B P65 Subunit
Scr	Scramble
siRNA	Small interfering RNA
Sir2	Silent information regulator 2
SIRT1	Sirtuin 1 (silent mating type information regulation 2, homolog 1)
STAC	Sirtuin activating compound
SREBP	Sterol regulatory element binding protein
TAMRA	Carboxytetramethylrhodamine
TNFα	Tumor necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule 1
VLDL-cholesterol	Very low-density lipoprotein cholesterol
WAT	White adipose tissue

3. Introduction

3.1 Atherosclerosis

Atherosclerosis is a chronic progressive disease of the arteries and is the leading cause of death in developed countries. Atherosclerosis occurs when excess circulating low-density lipoprotein cholesterol (LDL-cholesterol) is oxidised and modified to accumulate in the arteries triggering local inflammation and plaque formation. Such an accumulation over time leads to an increase in plaque size, leading to narrowing of the coronary arteries. However, it is not the size of the plaque itself but rather its ability to rupture and form a blood clot (thrombus) that is deadly. Histological analyses have shown that more than 40% of the volume of most disrupted plaques were occupied by a necrotic lipid core, consisting of lipids, cholesterol crystals and necrotic debris and surrounded by a thin fibrous cap.[1, 2]

In 1958 it was suggested that the pathological manifestation of plaque formation first occurred as an accumulation of fatty streaks.[3] These early 'fatty streak' lesions consist of T cells and lipid-loaded macrophage-like foam cells.[4] The pathogenesis is initiated by endothelial dysfunction, which permits sub-endothelial accumulation of excess low-density lipoprotein (LDL) particles containing cholesterol.[5] The retained subendothelial LDL particles are susceptible to oxidative stress and transform into so-called oxidized LDL (oxLDL) or minimally modified LDL (mmLDL) by yet unknown mechanisms.

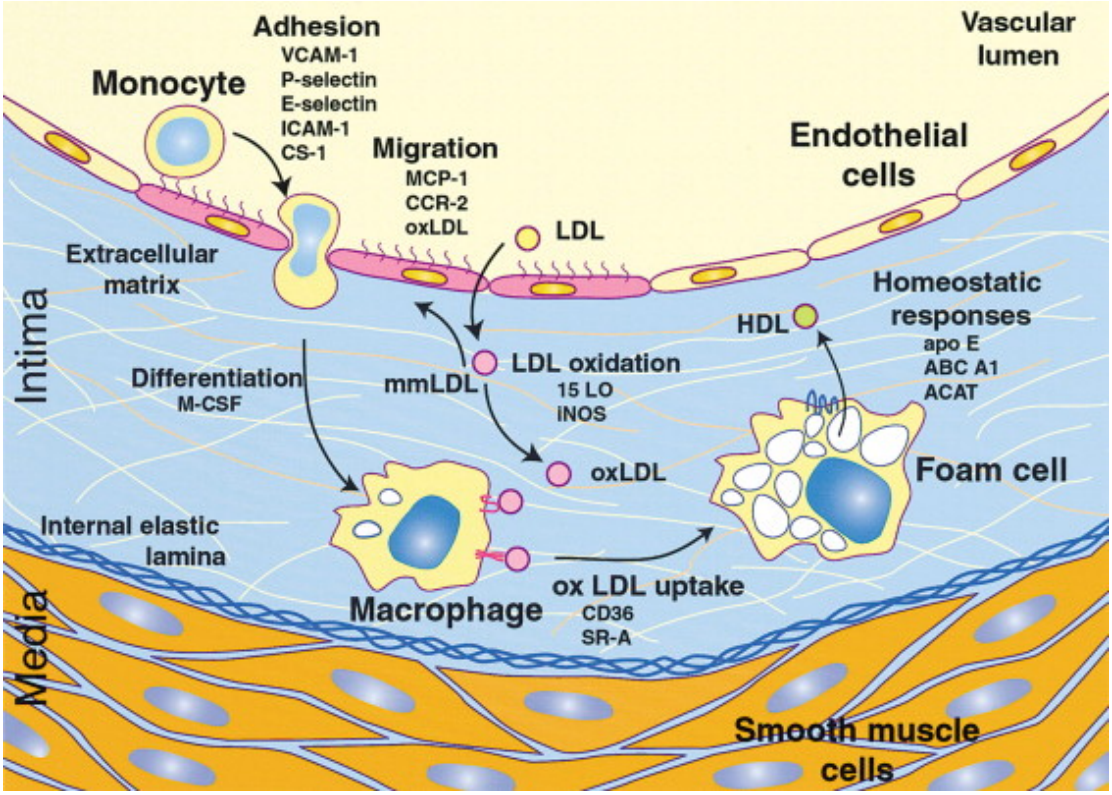

Figure 1. Formation of fatty streaks during atherosclerosis. *Excess LDL-cholesterol accumulates within the intima of the endothelium leading to endothelial activation and cytokine release. In the intima, these LDL particles are oxidized to form mmLDL or oxLDL. Macrophages ingest the oxLDL by scavenger receptors (CD36, SR-A) and accumulate to form foam cells. OxLDL is toxic to the cells and further causes the release of cytokines and chemokines from the endothelium, which increases the recruitment of monocytes leading to the formation of 'fatty streaks'.* (Adapted from Christopher K. Glass, Joseph L. Witztum, Cell, 2001)

High levels of mmLDL or oxLDL trigger endothelial cells to express adhesion molecules and release chemokines, facilitating the infiltration and activation of macrophages.[6] These activated macrophages phagocytise

oxLDL or mmLDL to form foam cells and fatty streaks beneath the endothelium. The excess oxidized lipid accumulation over time leads to the necrosis of foam cells and enhanced inflammation, thus leading to the formation of complex lesions. Since excess LDL-cholesterol is the main protagonist, lowering plasma LDL-cholesterol has evolved as an important therapy for treatment and prevention of atherosclerosis.

3.2 LDL-cholesterol and cardiovascular disease

Lipoprotein particles function as the main carriers of lipids in plasma. Low-density lipoprotein is one of five major groups of lipoproteins and is the most abundant cholesterol transporter in the human body. Unlike other lipoproteins (chylomicrons, very low-density lipoprotein, intermediate-density lipoprotein and high-density lipoprotein), due to its composition, LDL is highly susceptible to oxidation. An LDL particle is composed of tightly bundled cholesterol, surrounded by a hydrophilic surface layer of phospholipid, and a hepatic sourced ApoB100 protein that adds to its stability. The core of the particle consists of esterified and non-esterified cholesterol and triglycerides.

LDL-cholesterol has long been accepted as a major risk factor for the development of coronary heart disease (CHD) and is a well-validated target to reduce the risk of cardiovascular disease.[7] Early studies showed that reducing cholesterol biosynthesis by inhibiting its rate-limiting enzyme, 3-hydroxy-3-methylglutaryl Co-enzyme A (HMGCoA) reductase, reduces plasma LDL-cholesterol levels.[8] Thus the discovery of statins, which were potent inhibitors of HMGCoA reductase, ushered a new way to lower LDL-cholesterol. The

introduction of the first statin, Simvastatin, to patients in the Scandinavian Simvastatin Survival Study (4S) showed that lowering plasma cholesterol significantly reduced the risk of cardiovascular events in CHD patients.[9] A 1mmol/L reduction in LDL-cholesterol by statins was associated with a 20-25% reduction in coronary risk.[10] These findings proved that LDL-cholesterol lowering is an effective method to treat and prevent atherosclerosis.

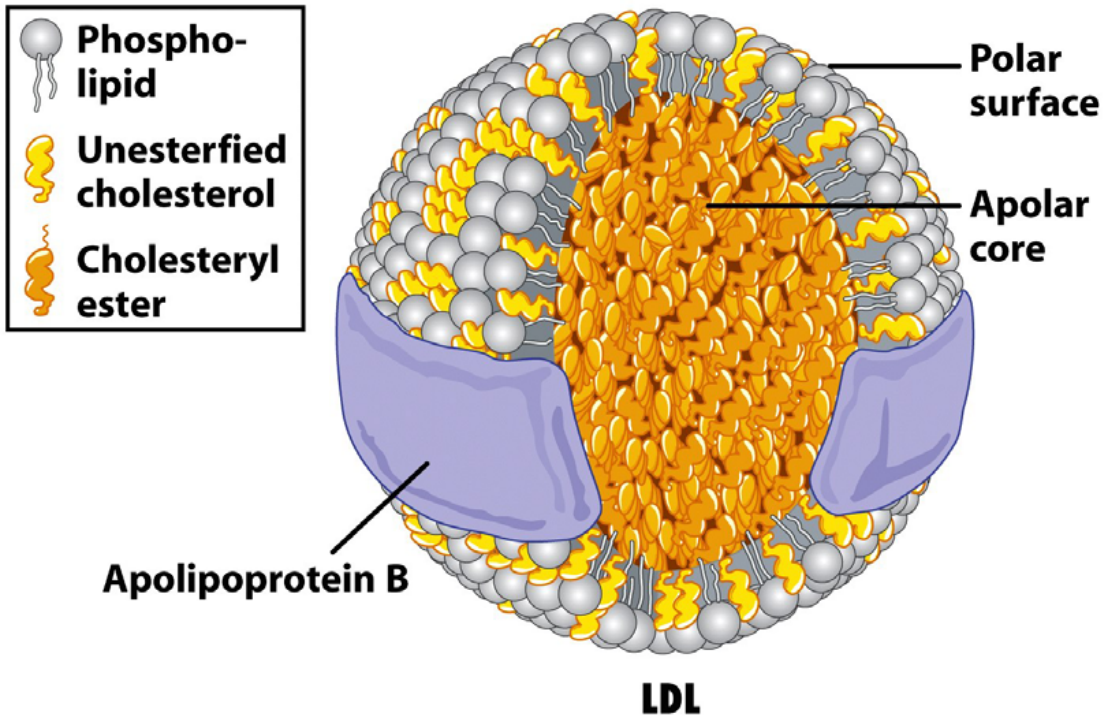

Figure 2. Cross-section of the low-density lipoprotein particle. *The LDL particle is a lipophilic sphere consisting of an exterior polar surface made up of triacylglycerols and cholesteryl esters and an interior apolar core consisting of phospholipids and unesterified free cholesterol. The sphere is surrounded by an apolipoprotein B-100 (ApoB100) molecule.* (Adapted from healthyprotocols.com)

According to the PROVE-IT trial, intensive lipid lowering statin regimens provided greater protection against death or major cardiovascular events, than a standard regimen[11], suggesting that continued lowering of LDL-cholesterol beyond target levels would improve risk events in patients with cardiovascular disease. Hence it is logical to assume that further reducing LDL-cholesterol beyond current statin-levels via a non-statin strategy could markedly lower the residual risk of events in patients already receiving a statin. However, increasing the dosages of statins and intensive statin therapy may have its limitations due to various side-effects, like myalgia (increased liver enzymes),[12] rhabdomyolysis (muscle breakdown),[13] sexual dysfunction or in some cases a risk of diabetes.[14, 15] Due to this the research and discovery of non-statin based LDL-cholesterol lowering drugs has attracted a lot of attention.

3.3 Regulation of LDL-cholesterol by Modulating the LDL receptor

Uptake of LDL by hepatic LDL receptors (LDL-R) is the primary mechanism for the clearance of circulating plasma LDL-cholesterol. Familial hypercholesterolemia (FH) is an autosomal dominant disease mainly caused by loss-of-function mutations in the gene encoding the LDL-R.[16] FH is characterized by reduced LDL-cholesterol clearance and thus, an increased risk of atherosclerosis. To date over 1100 mutations in the LDL-R gene have been identified in FH patients. The study of FH has been instrumental in the discovery and study of LDL-R in cholesterol metabolism and its related diseases. FH is known to exist in 2 forms, the less severe heterozygous form

(when the mutation is present in only one of the two alleles of the gene) and the more severe homozygous form (when the mutation is present in both alleles of the gene).[17] FH patients heterozygous for a mutation in the LDL-R gene have a 50% reduction in LDL-R, which causes a 2-fold increase in the number of LDL particles in blood from birth.[18] Similarly, FH patients homozygous for a mutation in the LDL-R gene have a LDL-cholesterol concentration in the range of 200 mg/dL to 700 mg/dL. In 1974, the lack of uptake of radiolabeled LDL – ^{125}Iodine-LDL in FH homozygote fibroblasts compared to normal fibroblasts, and the localisation of these radiolabeled LDL on the surface of normal fibroblasts led to the discovery of LDL-R.[19] Brown and Goldstein, who pioneered this study, further showed that the release of LDL from radioactive ^{125}I-LDL in normal cells caused the suppression of not only, 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoA reductase) gene, the rate limiting enzyme for cholesterol synthesis, but also the LDL-R gene. Such studies highlighted the critical importance of LDL-R in the metabolism and regulation of plasma cholesterol.

LDL-R is a transmembrane protein, 839 amino acids in length and consisting of 5 main domains, namely, the LDL-R repeat domain, the epidermal growth factor (EGF) repeat domain, O-linked glycosylation domain, transmembrane domain and the cytoplasmic domain.[20] The interaction between the LDL-R and circulating LDL is electrostatic in nature, with the acidic residues in the LDL-R binding domain interacting with the basic residues of the ligand – apoB100.[21] LDL-R is regulated by the transcription factor - sterol regulatory element binding protein (SREBP).[22] Low levels of

intracellular sterols cause a series of molecular events that cause the transcription factor to localise to the nucleus.[23] There are two genes encoding SREBP, with three different isoforms – SREBP1a, SREBP1c and SREBP2.[24] While SREBP1a and SREBP1c are involved in fatty acid metabolism, SREBP2 is the transcription factor mainly involved in cholesterol biosynthesis and metabolism. SREBP2 is the main transcription factor responsible for the transcription of HMGCoA reductase and LDL-R gene.[22] When intracellular lipids are low, the N-terminal of SREBP2 binds to sterol regulatory elements in the LDL-R promoter causing an increase in LDL-R expression,[25] which lead to an increase in surface-bound LDL-R expression. The LDL-R recognises the binding ligand, ApoB100, present on LDL particle through the LDL-R repeat domain. Recognition of the ApoB100 at neutral pH causes the internalisation of the LDL-LDL-R complex to form clathrin-coated vesicles.[26] These coated vesicles disperse into the endosome, in which the acidic pH of the endosome activates the release of internalized ligands from the receptor through the EGF-repeat domain. While the released ligands travel to the lysosome for its lipid content, the LDL-R recycles back to the cell surface. Thus LDL-R continues to take up circulating LDL-cholesterol, until excess internalised cholesterol causes a reduction in LDL-R transcription by inhibiting SREBP2. Another mechanism for cells to regulate LDL-R expression is through proprotein convertase subtilisin/kexin type 9 (PCSK9). PCSK9 is a secreted chaperone that binds to cell-bound LDL-R in the presence of LDL-cholesterol and after internalisation of the complex, targets it for lysosomal degradation.

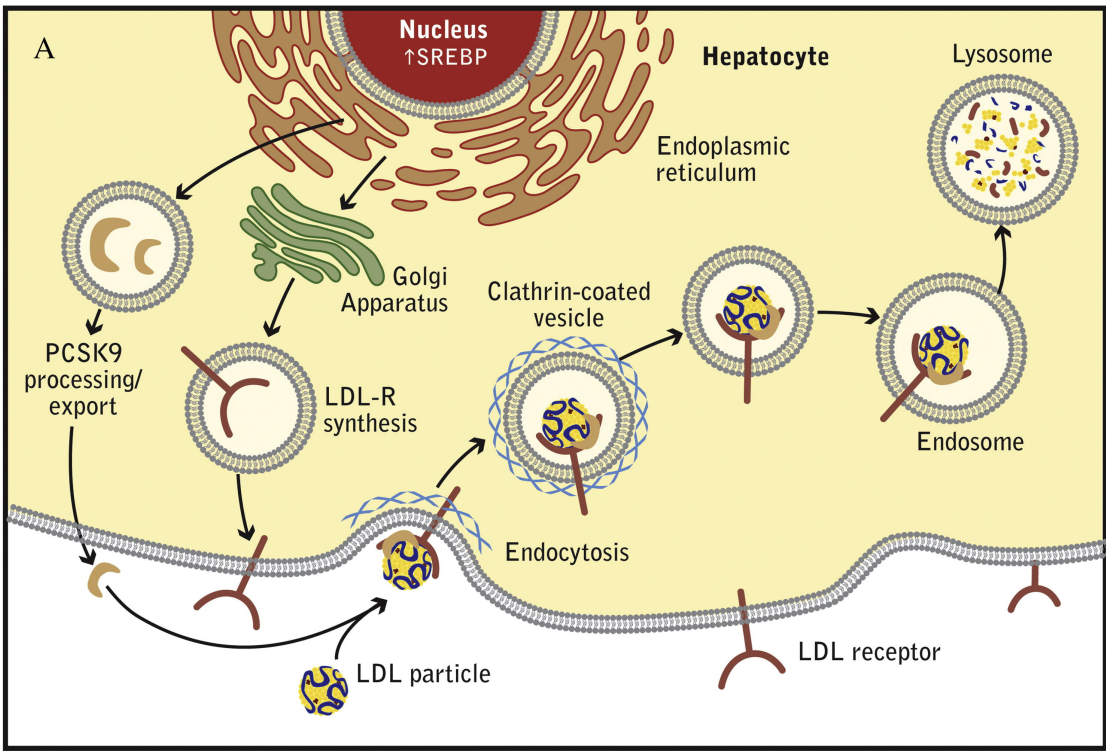

Figure 3. The role of PCSK9 in regulating expression of LDL-R. *Secreted PCSK9 binds to membrane-bound LDL-R. A complex of LDL-cholesterol, LDL-R and PCSK9 undergoes clathrin-mediated endocytosis and subsequently is degraded in the lysosome.* (Adapted from Catapano A.L and Papadopoulos N. Atherosclerosis, 2013).

3.4 LDL-R regulation by PCSK9

Proprotein convertase subtilisin/kexin type 9 is a member of the mammalian subtisilin family of proprotein convertases.[27] It is a 72-kDa chaperone predominantly expressed in the liver, intestine and kidney. PCSK9 contains four domains – an N-terminal signal domain, a prodomain, a catalytic domain and a cysteine-rich C-terminal domain. The precursor of PCSK9 undergoes autocatalytic cleavage of the 14-kDa prodomain in the endoplasmic reticulum, to form an active PCSK9 protein. Although the prodomain undergoes

cleavage, it does not detach from the active protein, but blocks its catalytic domain allowing PCSK9 to function as a chaperone. The active form of PCSK9 is then secreted into the plasma where it binds to the EGF domain of cell surface-bound LDL-R, in the presence of bound LDL-cholesterol. Internalised LDL-cholesterol-LDL-R-PCSK9 complex is then dispensed to the lysosome for degradation, thus preventing the recycling of LDL-R. Patients with gain-of-function mutations in PCSK9 have a 5- to 30-fold increased affinity for LDL-R compared to healthy subjects, thus having higher plasma LDL-cholesterol.[28] In line with this, patients with loss-of-function mutations in PCSK9 have lower levels of plasma LDL-cholesterol.[29]

Patients with gain-of-function mutations in PCSK9 have low sensitivity to statins with a predisposition to early onset atherosclerotic disease.[30] In addition to regulating LDL-R and HMGCoA reductase expression, the transcription factor SREBP2 is also responsible for the expression of PCSK9. Since SREBP2 is the common the transcription factor for all three genes, the expression of these proteins are known to be interdependent. Decreased cholesterol levels or pharmacological inhibition of HMGCoA reductase by statins activates SREBP2 transcriptional activity, which not only leads to an increase in LDL-R expression but also PCSK9 expression.[31] Consequently, statins increase plasma PCSK9 levels in a dose-dependent manner, thus limiting the LDL-cholesterol lowering effect of statins by an increase in PCSK9-mediated LDL-R degradation. These findings have stimulated the search for drugs that lower PCSK9 activity, serving as an adjunct to statins and/or as an alternative for treatment of atherosclerosis.

3.5 Caloric restriction and sirtuins

Caloric restriction (CR) refers to a metabolic state in which energy intake is reduced to below-normal levels, while being sufficiently high to avoid malnutrition. In 1935, Clive McCay for the first time described that restriction of calories without malnourishment prolongs mean and maximal lifespan in rats compared to ad libitum feeding.[32] He later recommended that CR might be used as a model to study aging, however, little was known about the mechanism of caloric restriction itself[33] Although Clive McCay and Otto Warburg described CR and the function of nicotinamide adenine dinucleotide (NAD^+) in the same decade, it was not until more than half a century later that Imai et al. described the requirement of NAD^+ for CR-dependent life-span extension in the yeast *S. Cerevisiae*.[34] Thus a new era of research began addressing the modulation of NAD^+ and NAD^+-dependent enzymes in aging and age-related diseases.

Coenzymatic activity of NAD^+, coupled with stringent regulation of its bioavailability, makes it an ideal candidate for a metabolic sensor. This notion is partly supported by the fact that NAD^+ functions as a substrate in key metabolic pathways such as the glycolytic pathway, respiratory chain and *de novo* biosynthesis of macromolecules. Metabolic homeostasis involves a sensitive balance between energy intake, utilization and storage. The discovery of the yeast enzyme – Silent information regulator 2 (Sir2) as a NAD^+-dependent deacetylase hinted at the importance of Sir2 in metabolic regulation.[34, 35] Although Sir2 is a NAD^+-dependent deacetylase, it does not

use NAD^+ as a redox acceptor. Sir2 cleaves the NAD^+ molecule during a deacetylation reaction, transferring the acetyl group to the ribose sugar, thereby producing O-acetyl-ADP-ribose and nicotinamide as by-products. The dependence of Sir2 activity on the cellular bioavailability of NAD^+ and its high K_m values for NAD^+ alluded to a likely link between Sir2 activity and the metabolic state of the cell.[35, 36] This led to studies investigating the relationship between aging and metabolic diseases involving Sir2 and Sir2-related proteins. In mammals the highly conserved Sir2 protein and its orthologs belong to a family of proteins called 'sirtuins'.

Sirtuins are a family of highly conserved, ubiquitously expressed, NAD^+-dependent class III histone deacetylase enzymes. They function mainly as metabolic sensors, translating changes in NAD^+ levels into adaptive responses. They are now known to deacetylate a wide number of transcriptional regulators and thus oversee a plethora of metabolic processes. Based on their sequences, mammalian sirtuins are divided into four classes – SIRT1 to SIRT3 belong to class I, SIRT4 to class II, SIRT5 to class III and SIRT6 and SIRT7 to class IV.[37] SIRT1 can shuttle between the nucleus and the cytoplasm, regulating gene expression by deacetylating nuclear transcription factors and cytoplasmic proteins,[38] while SIRT2 is localized to the cytoplasm.[39] SIRT3, SIRT4 and SIRT5 are mitochondrial proteins, whereas SIRT6 and SIRT7 are nuclear proteins, with SIRT7 mainly expressed in the nucleolus.[40-42] Not all sirtuins have the same enzymatic activity. SIRT1, SIRT2, SIRT3, SIRT6 and SIRT7 are known to mainly function as deacetylase enzymes, while SIRT4 functions as an ADP-ribosylation enzyme. SIRT5 on

the other hand, not only has deacetylase activity but also desuccinylase and demalonylase activity. Of the seven mammalian sirtuins, SIRT1 was the first to be discovered and has been best characterized for its metabolic effects.

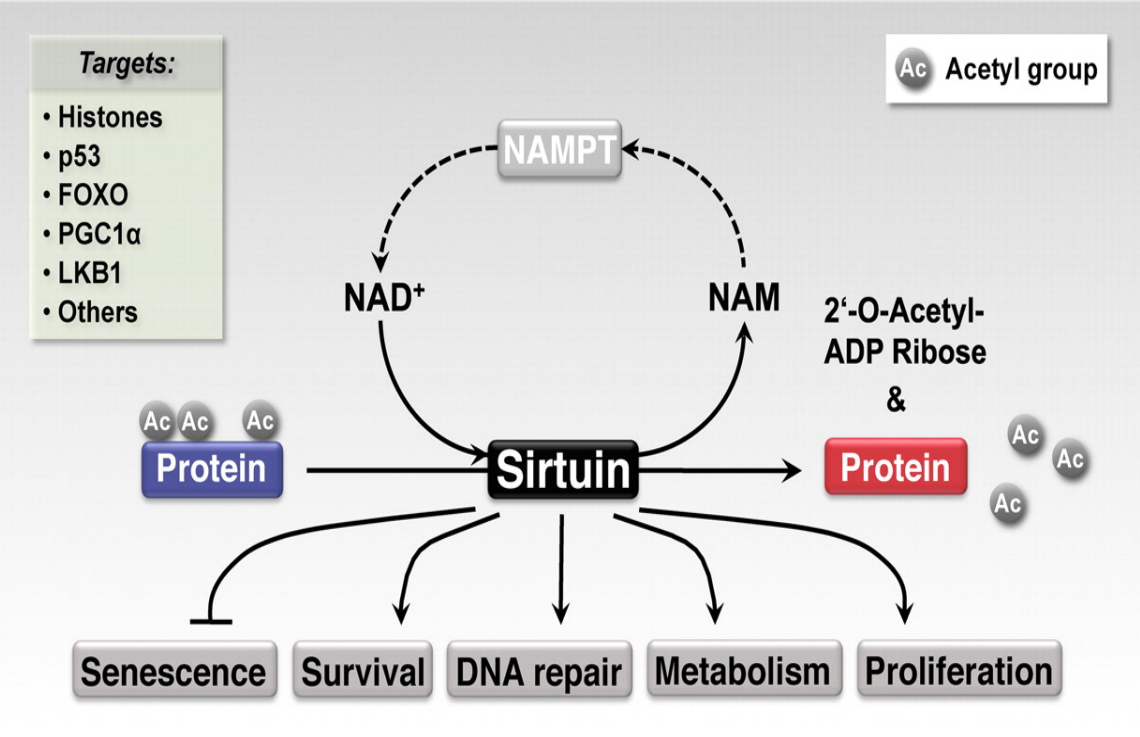

Figure 4. The Sirtuin family of protein deacetylases. *Sirtuins are NAD^+-dependent deacetylases that deacetylate multiple protein targets. In the deacetylation reaction, sirtuins utilize NAD^+ as a cofactor and produce a deacetylated protein, nicotinamide (NAM) and 2'-O-acetyl-ADP ribose. NAM is recycled back to NAD^+ by nicotinamide phosphoribosyltransferase (NAMPT).* (Adapted from Oellerich M F, and Potente M. Circulation Research, 2012)

3.6 SIRT1 and cellular metabolism

SIRT1 deacetylates the ε-acetyl-lysine residues of its target protein. Consistent with its dual localisation in the nucleus and cytoplasm, SIRT1 targets proteins in both cellular compartments. SIRT1 activity is generally accepted to increase with an increase in NAD^+ level. Since the K_m values of human SIRT1 are between 300-500μM,[43] which is well within the 0.2 to 0.5mM range of intracellular NAD^+ level,[44] cellular NAD^+ level may be the rate-limiting factor for SIRT1 enzymatic activity. Furthermore, although intracellular levels of NAD^+ are difficult to assess, they rarely fluctuate more than 2-fold, thus being sufficiently capable of modulating SIRT1 activity.[45, 46] Since both SIRT1 activity and NAD^+ levels, are known to increase during exercise, fasting or caloric restriction, it highlights the tight link between SIRT1 and the regulation of energy metabolism.[47, 48]

The importance of SIRT1 in normal development and physiology is highlighted by the fact that whole body deletion of SIRT1 is lethal. Inbred mice lacking both alleles of SIRT1 are smaller and die postnatal; outbred mice survive to adulthood and are sterile.[49] Murine whole-body overexpression of SIRT1 has been shown to display features mimicking the benefits of caloric restriction. SIRT1 overexpression in mice protects against high-fat diet-induced metabolic damage, insulin resistance and diabetes, with an increase in energy efficiency.[50, 51] However, there are two major drawbacks of SIRT1 overexpression studies. Firstly, higher SIRT1 expression may not necessarily result in increased SIRT1 activity; secondly, consistent overexpression of

SIRT1 may not resemble normal metabolic processes observed during caloric restriction. Braidy et al supported these drawbacks by demonstrating that rats having lower NAD^+ levels have a compromised level of SIRT1 activity.[52] Thus demonstrating that the activity of SIRT1 can adequately increase only in the presence of sufficient quantities of its bioavailable substrate, NAD^+, and not by solely overexpressing SIRT1 protein alone. Although SIRT1 was initially described as an enzyme that deacetylates mainly histones, it was later found to have many non-histone targets. p53 was the first non-histone target of SIRT1 to be discovered. SIRT1 deacetylates p53, inhibiting p53-mediated apoptosis, thus favouring cell repair and survival.[53] Subsequently, SIRT1 was shown to deacetylate numerous targets, including transcription factors like NFκB, PGC1α, LXR and different FOXO isoforms.[54-57]

3.7 SIRT1 and lipid metabolism

Sirtuins modulate multiple aspects of cellular lipid metabolism in a number of different tissues like white adipose tissue (WAT), liver and skeletal muscle SIRT1 has been shown to have affect WAT in different ways. Mice placed on a high-fat diet, overexpressing SIRT1 or treated with SIRT1 activators, show a reduction in the increase of WAT mass.[51, 58] SIRT1 gain of function mimics insulin-sensitizing function of PPARγ ligands *in vivo*.[50] SIRT1 deacetylates PPARγ in WAT promoting browning of subcutaneous WAT, thus promoting energy expenditure over energy storage.[59] Inhibition of SIRT1 in 3T3 L1 cells (an adipocyte differentiation model) promotes adipocyte differentiation while its overexpression blocks adipocyte differentiation.[60] Furthermore, SIRT1 also

promotes mobilization of free fatty acids from WAT by repressing PPARγ activity, an effect lost in SIRT1$^{-/-}$ mice.[60]

The liver is the main organ for the synthesis of cholesterol, free fatty acids and bile acids. Liver-specific deletion of SIRT1 results in an increase in hepatic steatosis and inflammation, while liver-specific overexpression of SIRT1 results in protection against high-fat diet-induced hepatic steatosis. In fasted mice, SIRT1 is induced in the liver where it deacetylates PGC1α resulting in the induction of gluconeogenic and fatty acid oxidation genes.[61] The expression of SIRT1 and PGC1α was shown to correlate with the intracellular concentrations of the metabolite - pyruvate, a metabolite associated with fasting.[56] These findings suggest that PGC1α undergoes cycles of acetylation and deacetylation during fed and fasted states, which correlate with SIRT1 expression. Loss of SIRT1 impairs the activity of hepatic PPARα due to lack of PGC1α deacetylation resulting in a decrease in fatty acid oxidation, thereby increasing steato-hepatitis in mice fed a high-fat diet.[62].

Currently, SIRT1 is recognized as a modulator of hepatic cholesterol levels by deacetylating LXR and PGC1α.[57, 61] Deacetylation of LXR leads to an increase in cholesterol efflux resulting from an increase in ABCA1 expression (a membrane transporter involved in HDL synthesis).[57] Increase in LXR activity by SIRT1 promotes reverse cholesterol transport and increases the synthesis of bile acids. In line with this, livers of SIRT1$^{-/-}$ mice have reduced mRNA levels of LXR target genes, including Cyp7A1, SREBP1c and fatty acid synthase.[63] Since excess cholesterol can be cleared from cells

through activation of SIRT1, many researchers have studied the role of SIRT1 in cardiovascular disease.[54-57]

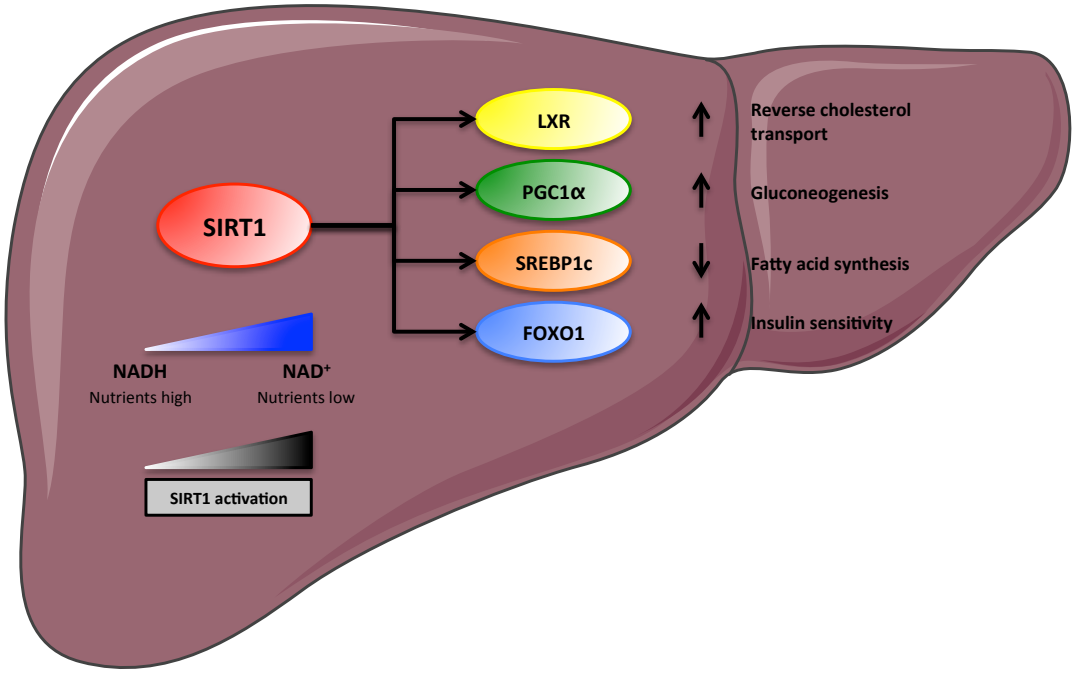

Figure 5. SIRT1 modulates multiple targets in the liver. *An increase in NAD^+ levels activates SIRT1 causing deacetylation of LXR, PGC1α, SREBP1c and FOXO1 which leads to an increase in reverse cholesterol transport, gluconeogenesis and insulin sensitivity with a decrease in fatty acid synthesis.*

3.8 SIRT1 and cardiovascular disease

Cardiovascular disease and aging pose a major risk to mortality in the developing world. Since the risk of coronary artery disease and congestive heart failure increase with age, SIRT1 has been extensively studied in cardiovascular disease. In aged mouse arteries, SIRT1 expression is reduced thus increasing the risk of endothelial damage.[64] SIRT1 has an extensive role

in protection against inflammation and cell survival by acting as a potent anti-senescence and anti-inflammatory protein in different tissues through various mechanisms. In the endothelium, SIRT1 expression is very abundant and functions as a novel modulator of angiogenesis.[65] SIRT1 does this by deacetylating the angiogenesis-regulator FOXO1, resulting in endothelial remodelling and maintenance through an increase in FOXO1 target gene expression.

Endothelial dysfunction is involved in the formation of early lesions in atherosclerosis and is well established as a cardiovascular risk. Thus understanding the role of SIRT1 in endothelial dysfunction is key to discerning the benefits of SIRT1 activation in atherosclerosis. SIRT1 was found to negatively regulate p66 expression, a key player in endothelial dysfunction, through epigenetic modification.[66] Downregulation of p66 in the endothelium protects against hyperglycaemia-induced endothelial dysfunction. Another hallmark of endothelial dysfunction is the reduction of eNOS activity and expression.[67] The activity and expression of eNOS directly correlates with the activity and/or expression of SIRT1. SIRT1 deacetylates eNOS in the endothelium suggesting that SIRT1 impedes endothelial dysfunction by increasing the activity of eNOS.[68] In support of this, endothelium-specific overexpression of SIRT1 protects against atherogenesis in mice fed a high-fat diet by promoting endothelium-dependent vasodilation through eNOS. SIRT1 also inhibits oxLDL-induced endothelial cell apoptosis *in vitro*.[69] Furthermore, SIRT1 can downregulate inflammation by deacetylating and inhibiting p65 activity.[70] Deacetylation of p65 lead to suppression of chemokine secretion

and downregulation of scavenger receptors in macrophages, thus reducing endothelial activation and foam cell formation.[71, 72]

Cyclin-dependent kinase 5 (CDK5) is a kinase that blocks the activity of SIRT1 by phosphorylating SIRT1 at serine 47 position. In favour of an atheroprotective function of SIRT1, prevention of CDK5-mediated hyperphosphorylation of SIRT1 by Roscovitine, a CDK5 inhibitor, prevents the development of atherosclerosis in $ApoE^{-/-}$ mice.[73] Similar to macrophages, SIRT1 plays a protective role in vascular smooth muscle cells as well. Vascular smooth muscle cell-specific overexpression of SIRT1 increases DNA repair by deacetylating and activating Nijmegen Breakage Syndrome-1 protein. In support of this, deletion of SIRT1 in vascular smooth muscle cells of $ApoE^{-/-}$ mice showed an increase in oxLDL-induced DNA damage leading to atherogenesis and plaque formation.[74]

The role of SIRT1 has been investigated in patients suffering from cardiovascular disease as well. Human atherosclerotic plaques have reduced mRNA and protein expression of SIRT1 compared to normal vessels of patients with coronary artery disease.[74] SIRT1 expression in monocytes is reduced in patients with stable coronary artery disease and acute coronary syndrome.[75] Moreover, patients with mutations of SIRT1 gene (haplotype 2 carriers) have been shown to have a reduced risk of cardiovascular disease,[76] Thus, these findings suggest that increasing SIRT1 expression and activity in patients suffering from atherosclerosis may have a therapeutic potential in humans.

3.9 SIRT1 activators in animal studies

On the rationale that caloric restriction activates SIRT1 and has positive effects on health, the discovery of SIRT1 activators has provided a novel approach to mimic the benefits of caloric restriction in patients. Resveratrol was the first synthetic sirtuin activating compound (STAC), and still the most potent, natural-occurring compound found to activate SIRT1 *in vitro*. By activating SIRT1, resveratrol reduces ischaemic injuries in various tissues including the brain, heart and kidney, and improves vascular function.[77, 78] These protective effects were shown to involve eNOS activation and a reduction in cytokine production. Resveratrol reduces LPS-induced inflammatory responses of macrophages and peripheral blood monocytes *in vitro*.[79] Administration of resveratrol to patients at risk of CVD led to reduced levels of oxLDL and an improved lipid profile.[80] Furthermore, resveratrol mimicked the beneficial effects of SIRT1 *in vivo*, consistent with animal models overexpressing SIRT1.

Later on, however, resveratrol was shown to have SIRT1-independent effects, which were shown to involve activation of AMPK.[81] The discovery of synthetic STACs, distinct from resveratrol brought new hope for SIRT1 activation as a therapeutic target in a clinical setting. The STAC, SRT1720, was shown to reduce energy expenditure and lower plasma total cholesterol and LDL-cholesterol levels in obese mice, thus highlighting a potential role for SRT1720 as an LDL-cholesterol lowering agent.[82] Similarly, SRT1720 was shown to reduce the incidence of myocardial infarction.[83]

3.10 SIRT1 activators and their specificity

Although the effects of STACs are well documented, the mechanism by which STACs activate SIRT1 is still controversial. Originally STACs were thought to activate SIRT1 by lowering its Michaelis constants (K_m) for both SIRT1 protein substrates and NAD^+, thus allowing for an increase in deacetylase activity. Since then, there has been a lot of speculation that the published SIRT1 activators do not directly bind to and activate SIRT1 but act via an indirect mechanism through other proteins.

The first lead molecule to activate SIRT1 - resveratrol, was indeed shown to activate SIRT1 indirectly through AMPK activation.[81] The study showed that the AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) increased NAD^+ levels and caused an increase in PGC1α deacetylation in a SIRT1-dependent manner. Thus, the validity of resveratrol as a genuine SIRT1 activator was questioned. Furthermore, the initial assay to determine natural activators was a fluorometric assay called 'Fluor de Lys', in which acetylated peptide substrates of SIRT1 were non-covalently conjugated to aminomethylcoumarin (AMC), while synthetic activators were assessed by fluorescence polarization assay with carboxytetramethylrhodamine (TAMRA) –conjugated substrates and verified by mass spectrometry. Due to the large size of the fluorophores conjugated to SIRT1 substrates, this strategy of assessing STACs was highly criticized. Using NMR, surface plasmon resonance, and isothermal calorimetry techniques, Pacholec et al. proved that the STACs at that time - SRT1720, SRT2183 and SRT1460 and resveratrol, directly interact with the fluorophore-

containing peptide substrates of the SIRT1 activity assay.[84] Thus, they showed that the SIRT1 activity of these synthetic activators was an *in vitro* artefact. Such findings questioned the validity of this SIRT1 activity assay to detect novel SIRT1 activators and assess the SIRT1 activation potency of these drugs.

However, recent studies have vindicated the SIRT1 activity assay by showing that activation of SIRT1 by STACs is also dependent on the structural features of the peptide substrate.[85] Furthermore, another study showed that the fluorophore tags conjugated to the substrates used in the assay mimic the hydrophobic amino acids of natural substrates at the same position as the fluorophore. Thus this study suggested that synthetic SIRT1 activators discovered by the Fluor de Lys, or TAMRA assay tend to selectively deacetylate SIRT1 targets that have large hydrophobic residues (like Trp, Tyr or Phe) at the same position (eg. PGC1α-K778 and Foxo3a-K290).[86] Thus current SIRT1 activators indeed tend to activate SIRT1 by allosteric binding, however, they cause the deacetylation of only selected substrates. Accordingly, SIRT1 activators indeed activate SIRT1. SRT3025 is a new SIRT1 activator, discovered by the same process and is considered to have a similar mechanism of SIRT1 activation. SRT3025 causes an increase in activation of wild-type SIRT1 but fails to activate a SIRT1 mutant resistant to activation (E230K in which Glu^{230} is mutated to Lys). The beneficial effects of SIRT1 activators like SRT3025 have yet to be assessed in atherosclerosis.

4. Hypothesis, aims and design

4.1 Hypothesis

Based on our previous findings and recent observations described above, we hypothesize that,

Sirt1 activation by SRT3025 protects ApoE$^{-/-}$ mice against atherosclerosis by reducing inflammation and lowering plasma low-density lipoprotein cholesterol.

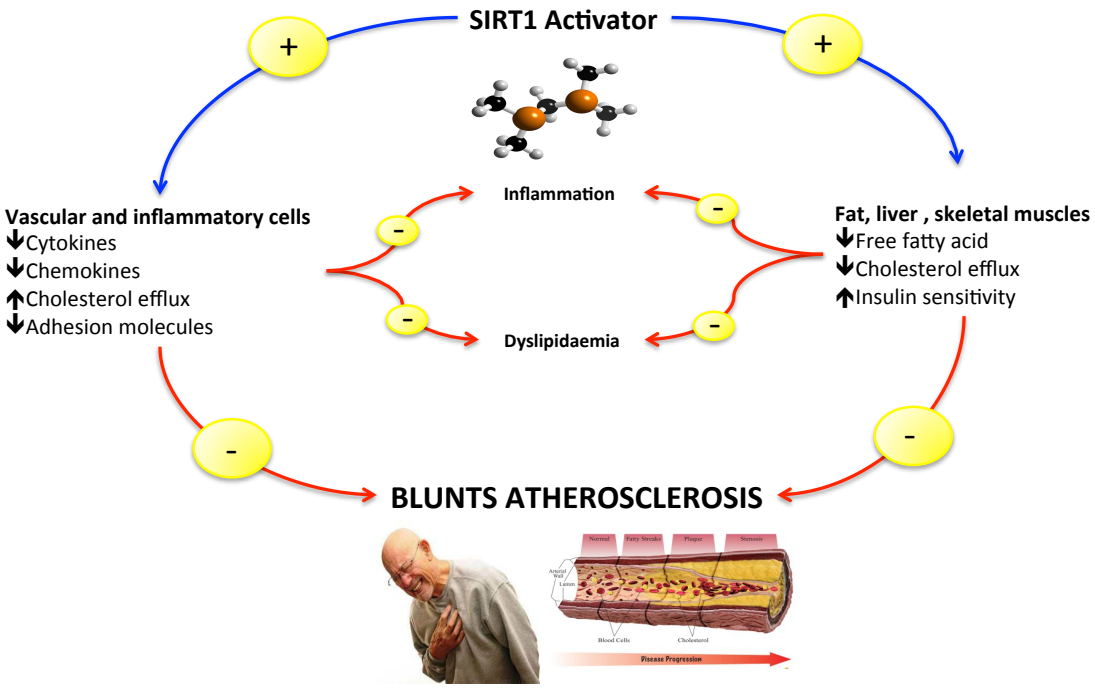

Figure 6. Overview of project hypothesis. *SIRT1 activation by an activator reduces inflammation by decreasing cytokine and adhesion molecules and improves lipid dysfunction by increasing cholesterol efflux, insulin sensitivity and inhibiting free fatty acid synthesis. The inhibition of inflammation and improvement of plasma lipid profile, both contribute to a decrease in atherosclerosis.*

4.2 Specific aims

Based on the above hypothesis, we formulated the following specific aims,

Aim 1: To assess the effects of SIRT1 activator SRT3025 on atherosclerosis in ApoE$^{-/-}$ mice fed a high-cholesterol diet.

Aim 2: To assess the effects of SIRT1 activator SRT3025 on inflammation in ApoE$^{-/-}$ mice fed a high-cholesterol diet.

Aim 3: To assess the effects of SIRT1 activator SRT3025 on plasma cholesterol levels in ApoE$^{-/-}$ mice fed a high-cholesterol diet.

4.3 Experimental design

8-week old ApoE$^{-/-}$ mice were fed a high-cholesterol diet (with 1.25% cholesterol) and supplemented with and without SRT3025 (3.18g/kg diet) for 12 weeks.

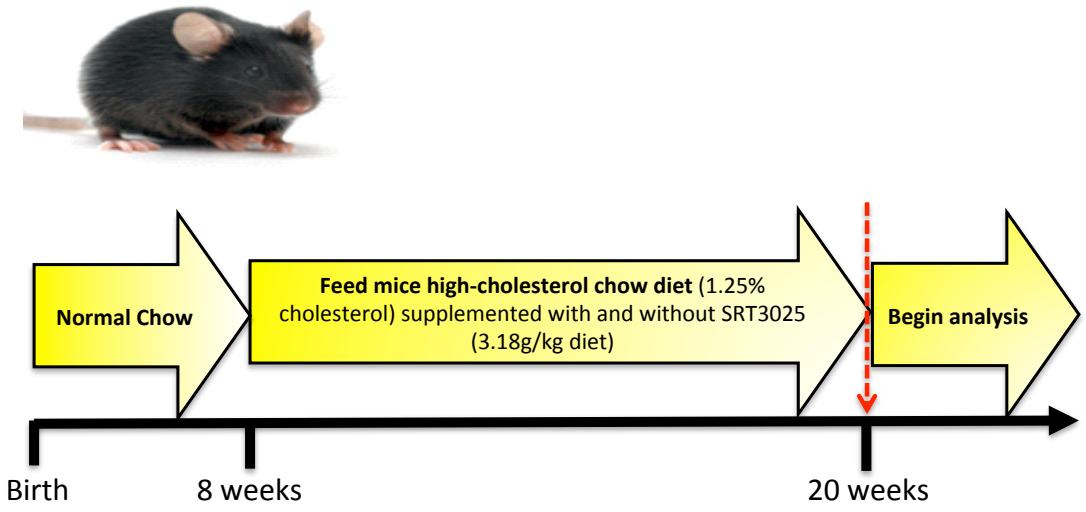

Figure 7. Overview of the experimental design for experimental atherosclerosis. *8-week old ApoE$^{-/-}$ mice on a normal chow were placed on a 1.25% high-cholesterol diet supplemented with and without SIRT1 activator, SRT3025 (3.18g/kg diet) for 12 weeks. After 12 weeks the mice were sacrificed and assessed for atherosclerosis.*

5. Results

5.1 Primary publication

The Sirt1 activator SRT3025 in *Apoe*$^{-/-}$ mice provides atheroprotection by reducing hepatic Pcsk9 secretion and enhancing LDL-R expression.

Authors Miranda MX, van Tits LJ, Lohmann C, Arsiwala T, Winnik S, Tailleux A, Stein S, Gomes AP, Suri V, Ellis JL, Lutz TA, Hottiger MO, Sinclair DA, Auwerx J, Schoonjans K, Staels B, Lüscher TF, Matter CM.

Journal Eur Heart J. 2014, *fasttrack* publication

PMID 24603306

Contributions: Experimental design, writing and revisions.

Experiments performed for

1. Figure 1. A, B, C, D and G
2. Figure 2. B and C
3. Figure 3. A to C
4. Figure 4. A to E
5. Figure 5. A, B
6. Figure 6. A, B
7. Figure 7. A, B, C, D and G

5.2 Contributions to other published articles

Deletion of Sirt3 does not affect atherosclerosis but accelerates weight gain and impairs rapid metabolic adaptation in LDL receptor knockout mice: implications for cardiovascular risk factor development.

Authors Winnik S, Gaul DS, Preitner F, Lohmann C, Weber J, Miranda MX, Liu Y, van Tits LJ, Mateos JM, Brokopp CE, Auwerx J, Thorens B, Lüscher TF, Matter CM.

Journal Basic Res Cardiol. 2014

PMID 24370889

Contribution: Input on experimental design and interpretation on Figure 5 and Figure 6.

Endothelial mineralocorticoid receptor activation mediates endothelial dysfunction in diet-induced obesity.

Authors Schäfer N, Lohmann C, Winnik S, van Tits LJ, Miranda MX, Vergopoulos A, Ruschitzka F, Nussberger J, Berger S, Lüscher TF, Verrey F, Matter CM.

Journal Eur Heart J. 2013

PMID 23594590

Contribution: Statistical analysis, revisions for Figure 3. A to D.

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FASTTRACK BASIC SCIENCE

The Sirt1 activator SRT3025 provides atheroprotection in *Apoe*$^{-/-}$ mice by reducing hepatic Pcsk9 secretion and enhancing Ldlr expression

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Aims The deacetylase sirtuin 1 (Sirt1) exerts beneficial effects on lipid metabolism, but its roles in plasma LDL-cholesterol regulation and atherosclerosis are controversial. Thus, we applied the pharmacological Sirt1 activator SRT3025 in a mouse model of atherosclerosis and in hepatocyte culture.

Methods and results *Apolipoprotein E*-deficient (*Apoe*$^{-/-}$) mice were fed a high-cholesterol diet (1.25% w/w) supplemented with SRT3025 (3.18 g kg^{-1} diet) for 12 weeks. *In vitro*, the drug activated wild-type Sirt1 protein, but not the activation-resistant Sirt1 mutant; *in vivo*, it increased deacetylation of hepatic p65 and skeletal muscle Foxo1. SRT3025 treatment decreased plasma levels of LDL-cholesterol and total cholesterol and reduced atherosclerosis. Drug treatment did not change mRNA expression of hepatic LDL receptor (Ldlr) and proprotein convertase subtilisin/kexin type 9 (Pcsk9), but increased their protein expression indicating post-translational effects. Consistent with hepatocyte Ldlr and Pcsk9 accumulation, we found reduced plasma levels of Pcsk9 after pharmacological Sirt1 activation. *In vitro* administration of SRT3025 to cultured AML12 hepatocytes attenuated Pcsk9 secretion and its binding to Ldlr, thereby reducing Pcsk9-mediated Ldlr degradation and increasing Ldlr expression and LDL uptake. Co-administration of exogenous Pcsk9 with SRT3025 blunted these effects. Sirt1 activation with SRT3025 in *Ldlr*$^{-/-}$ mice reduced neither plasma Pcsk9, nor LDL-cholesterol levels, nor atherosclerosis.

Conclusion We identify reduction in Pcsk9 secretion as a novel effect of Sirt1 activity and uncover Ldlr as a prerequisite for Sirt1-mediated atheroprotection in mice. Pharmacological activation of Sirt1 appears promising to be tested in patients for its effects on plasma Pcsk9, LDL-cholesterol, and atherosclerosis.

Keywords Sirt1 • LDL-cholesterol • Pcsk9 • LDL receptor • Atherogenesis

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Translational perspective

The deacetylase Sirt1 exerts beneficial effects on metabolic and inflammatory diseases. However, its effects on lipid metabolism and atherosclerosis remain controversial. Lowering plasma Pcsk9 emerges as a promising therapeutic strategy to lower plasma LDL-cholesterol. Unlike current antibody-based strategies that act on extracellular Pcsk9 activity, we found an alternative route to decrease Pcsk9-mediated Ldlr degradation: pharmacological Sirt1 activation reduced hepatic Pcsk9 secretion, increased Ldlr expression, and decreased plasma LDL-cholesterol and atherosclerosis in mice. Thus, Sirt1 activation appears as a promising approach to be tested for atheroprotection in patients.

Introduction

Atherosclerosis results from a complex interplay between innate and adaptive immunity involving modified LDL-cholesterol, activated endothelial cells, and monocyte-derived macrophages within the arterial wall. Sirt1 is a member of the sirtuin family of NAD^+-dependent deacetylases.[1] Studies on the role of Sirt1 in atherosclerosis have reported controversial effects.

Sirt1 has been shown to be atheroprotective in apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice[2–4] whereas genetic overexpression of Sirt1 in LDL-receptor-deficient (*Ldlr*$^{-/-}$) mice enhanced atherosclerosis.[5] The role of Sirt1 in regulation of plasma LDL-cholesterol concentration, the key trigger of atherogenesis,[6] remains incompletely understood. Hepatic deletion of Sirt1 in C57BL/6 mice fed a high-cholesterol diet induced mild hypercholesterolemia.[7] Conversely, administration of a Sirt1 activating drug to elderly volunteers and cigarette smokers decreased plasma levels of total and LDL-cholesterol.[8,9] These studies suggest that Sirt1 regulates plasma LDL-cholesterol.

Hepatic Ldlr clears LDL-cholesterol from the blood stream.[10,11] Transcription of Ldlr is controlled by the sterol-responsive element binding protein 2 (Srebp2),[12] while its turnover depends on proprotein convertase subtilisin/kexin type 9 (Pcsk9), a serine protease.[13] Secreted Pcsk9 targets hepatic Ldlr for lysosomal degradation and thus prevents recycling of internalized Ldlr to the cell surface.[14]

We hypothesized that hepatic Ldlr mediates the effects of Sirt1 on plasma LDL-cholesterol levels and thus provides atheroprotection. To test this hypothesis, we fed *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice a high-cholesterol diet supplemented with the novel Sirt1 activator SRT3025 or placebo, and investigated atherogenesis and lipid metabolism.

Methods

Detailed information is available in Supplementary material online.

Animals

Male *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice on a pure C57BL/6J background were housed with a 12 h light–dark cycle and fed a high-cholesterol diet containing 1.25% cholesterol (D12108; Research Diets) supplemented with or without (SRT3025, 3.18 g kg^{-1} diet, provided by Sirtris, Cambridge, MA, USA) for 12 weeks starting at the age of 8 weeks. After this treatment period, mice were sacrificed (after overnight fasting), EDTA blood was taken and tissues were harvested. All experiments and animal care procedures were approved by the local veterinary authorities and carried out in accordance with our institutional guidelines.

Cell culture

AML12 mouse hepatoma cells were cultured in a 1:1 (v/v) mixture of DMEM and Ham's F12 medium supplemented with insulin (5 μg ml^{-1}), transferrin (5 μg ml^{-1}), selenium (5 ng ml^{-1}), dexamethasone (40 ng ml^{-1}), and 10% FBS (v/v). Where indicated, AML12 cells were exposed to 10 μM SRT3025 in 1% DMSO (v/v).

Statistics

All data are presented as means ± SD. Data distribution was assessed using the Kolmogorov–Smirnov test. Normally distributed data were compared by an unpaired two-tailed Student's *t*-test; for non-parametric data the Mann–Whitney test was used. Three or more groups were compared using a Kruskal–Wallis test followed by a Dunn's post-hoc comparison (non-parametric data). At least three independent experiments in triplicates were performed. Significance was accepted at $P < 0.05$. Analyses were done using Graphpad Prism (version 5.0d, 2010).

Results

SRT3025 reduces plasma cholesterol, inflammation, and atherosclerosis in Apoe$^{-/-}$ mice

Histomorphometry of thoraco-abdominal aortae *en face* and cross-sections of aortic roots revealed a significant reduction in plaque size in SRT3025-treated *Apoe*$^{-/-}$ mice compared with placebo-treated controls (*Figure 1A* and *B*). Moreover, a marked reduction of Cd68-positive macrophages within the plaque and Vcam-1 expression in aortic roots was observed (*Figure 1C* and *D*). Interestingly, plasma levels of total-, LDL- and VLDL-cholesterol were significantly lower after SRT3025 treatment compared with placebo (*Figure 1E* and *F*). Triglycerides and HDL-cholesterol remained unchanged (Supplementary material online, *Figure S1A*). In addition, we observed reduced plasma levels of Mcp-1 and Il-6 (*Figure 1G*) and lower hepatic mRNA expression of these cytokines (*Figure 3A*).

These findings indicate that SRT3025 administration to *Apoe*$^{-/-}$ mice provides atheroprotection and reduces plasma LDL-cholesterol and inflammation.

SRT3025 mimics Sirt1 activity in vitro and in Apoe$^{-/-}$ mice

SRT3025 concentration-dependently activated wild-type Sirt1, but failed to activate the activation-resistant Sirt1 mutant E230K *in vitro* (*Figure 2A*). Increased deacetylation of known Sirt1 targets upon SRT3025 treatment, hepatic p65 and forkhead transcription factor family O1 (Foxo1) in skeletal muscle (*Figure 2B* and *C*) indicate successful Sirt1 activation *in vivo*. SRT3025 prevented an increase in weight gain and epididymal white adipose tissue without affecting food intake, thereby mimicking a caloric restriction phenotype

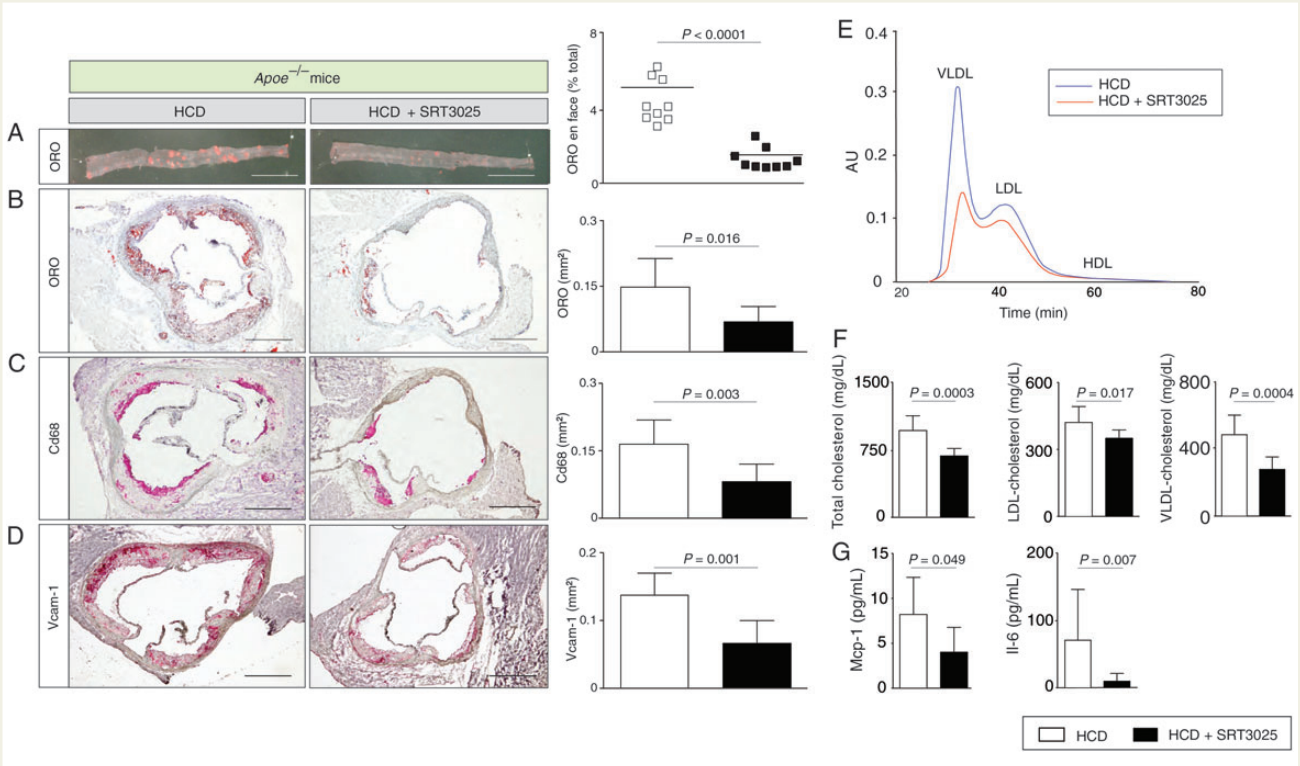

Figure 1 SRT3025 confers atheroprotection, reduces plasma cholesterol and systemic inflammation in Apoe$^{-/-}$ mice. Eight-week-old *Apoe*$^{-/-}$ mice were fed a high-cholesterol diet (1.25% w/w) supplemented with the Sirt1 activator SRT3025 ($n = 9$) or placebo ($n = 9$) for 12 weeks. Representative micrographs (left) and quantifications (right) of thoraco-abdominal aortae *en face* (*A*) and of aortic root cross sections (*B*–*D*) stained with ORO or immunohistochemically for macrophages (Cd68) or Vcam-1; (*E*) cholesterol distribution in the different lipoprotein subfractions separated by gel filtration chromatography; (*F*) Plasma total cholesterol, LDL-, and VLDL-cholesterol concentrations; (*G*) Plasma levels of Mcp-1 and Il-6. Scale bars in photomicrographs: 1 mm (*A*) and 500 μm (*B*–*D*). HCD, high-cholesterol diet; AU, arbitrary units; LDL, low-density lipoprotein; ORO, oil-red O; VLDL, very low-density lipoprotein; Vcam-1, vascular cell adhesion molecule 1; Mcp-1, monocyte chemoattractant protein 1; Il-6, interleukin 6.

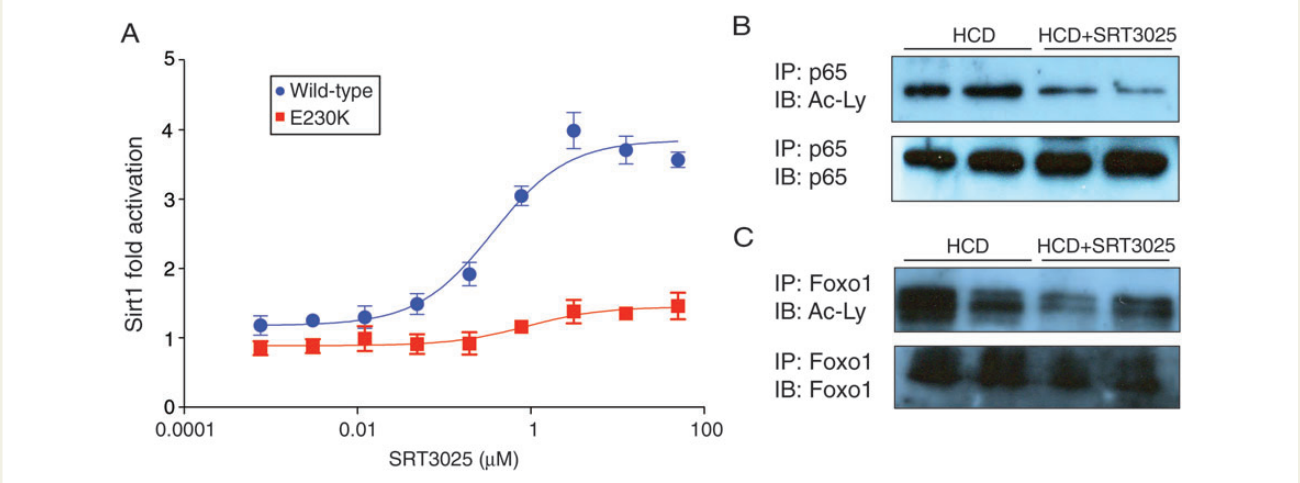

Figure 2 SRT3025 mimics Sirt1 activity *in vitro* and in Apoe$^{-/-}$ mice. (*A*) Concentration-response curve of SRT3025 on the activity of wild-type Sirt1 and activation-resistant mutant E230K *in vitro*. (*B*, *C*) Western blots for acetylation status of Sirt1 target proteins (*B*) p65 and (*C*) Foxo1 immunoprecipitated from nuclear extracts of liver and skeletal muscle, respectively, from *Apoe*$^{-/-}$ mice fed a high-cholesterol diet (1.25% w/w) supplemented with the Sirt1 activator SRT3025 or placebo for 12 weeks. HCD, high-cholesterol diet; Ac-Ly, anti-acetyl-lysine antibody.

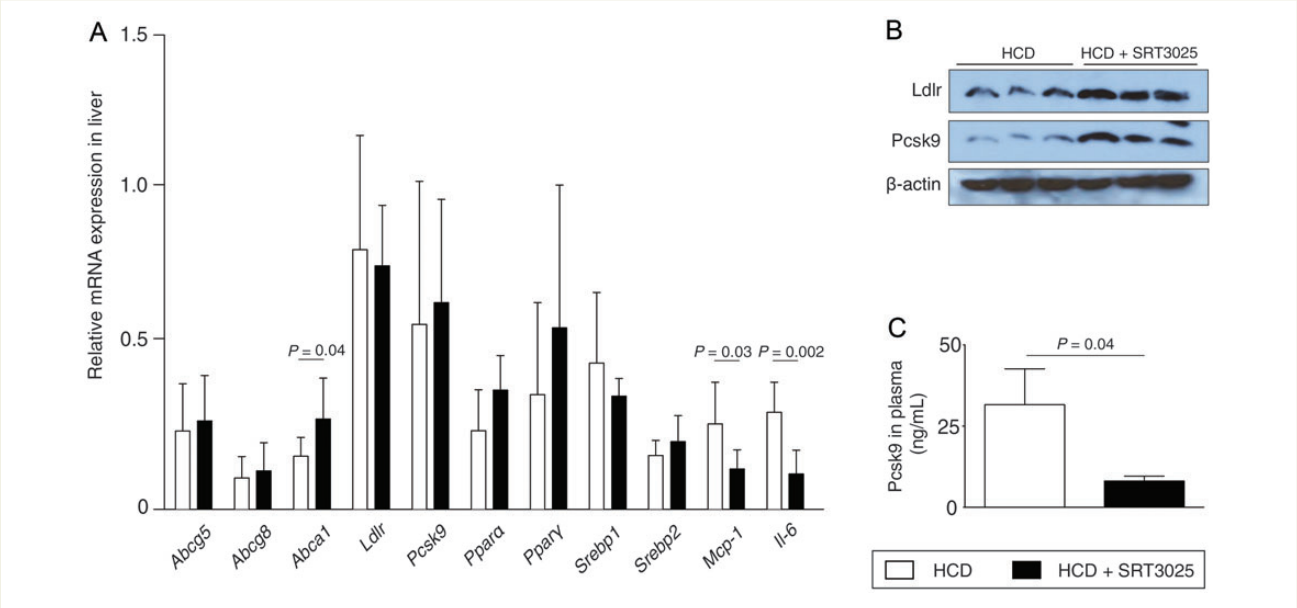

Figure 3 SRT3025 increases hepatic Ldlr protein expression while decreasing plasma Pcsk9 in Apoe$^{-/-}$ mice. Eight week-old *Apoe*$^{-/-}$ mice were fed a high-cholesterol diet (1.25% w/w) supplemented with the Sirt1 activator SRT3025 ($n = 9$) or placebo ($n = 9$) for 12 weeks. (*A*) Relative mRNA expression levels of hepatic genes involved in cholesterol regulation. (*B*) Western blots of liver lysates for Ldlr, Pcsk9, and β-actin. (*C*) Plasma levels of Pcsk9. HCD, high-cholesterol diet; Pcsk9, proprotein convertase subtilisin/kexin type 9.

(Supplementary material online, *Figure S1B–D*). Pharmacokinetic analysis of the drug in *Apoe*$^{-/-}$ mice showed that the drug indeed reached target tissues (Supplementary material online, *Table S1*). Plasma protein levels of glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, and alkaline phosphatase were not different between drug- and placebo-treated mice (Supplementary material online, *Figure S1E*).

SRT3025 increases hepatic Ldlr expression and Pcsk9 accumulation in Apoe$^{-/-}$ mice

Pharmacological Sirt1 activation increased liver expression of *Abca1*, but did not affect *Abcg5*, *Abcg8*, *Ldlr*, *Pcsk9*, *Pparα*, *Pparγ*, *Srebp1*, and *Srebp2* (*Figure 3A*). Despite no changes in *Ldlr* and *Pcsk9* mRNA expression, both were markedly increased at the protein level (*Figure 3A* and *B*). This intracellular accumulation of Pcsk9 protein with no change in mRNA suggests a disturbed transport and/or secretion of the protein. In agreement with this, we found reduced plasma Pcsk9 protein levels following pharmacological Sirt1 activation in *Apoe*$^{-/-}$ mice compared with placebo-treated controls (*Figure 3C*).

SRT3025 increases Ldlr expression and Pcsk9 accumulation in AML12 hepatocytes

To delineate the mechanisms by which SRT3025 affects Ldlr protein expression, we performed *in vitro* experiments administering SRT3025 to mouse hepatoma AML12 cells. We observed a concentration- and time-dependent increase in Ldlr and Pcsk9 protein expression upon SRT3025 administration in cell lysates (*Figure 4A* and *B*). As observed *in vivo*, mRNA levels of *Ldlr* and *Pcsk9* were not altered by SRT3025 (*Figure 4C*), indicating that post-translational effects cause the changes in protein expression.

Incubation of AML12 cells with 10 μM SRT3025 was associated with a time-dependent decrease in Pcsk9 secretion into the supernatant (*Figure 4D*). Moreover, co-immunoprecipitation of endogenous Pcsk9 and Ldlr after 24 h incubation with 10 μM SRT3025 revealed that Pcsk9 binding to Ldlr was impaired after SRT3025 treatment compared with vehicle control (*Figure 4E*). Thus, the question arises whether the increase in hepatic Ldlr protein expression upon Sirt1 activation is related to limited extracellular availability of Pcsk9 and/or a defective degradation of internalized Ldlr.

SRT3025 impairs Pcsk9-dependent degradation of Ldlr in AML12 hepatocytes

To address the above question, we stimulated AML12 cells with 10 μM SRT3025 for 24 h and compensated for the decrease in extracellular Pcsk9 by adding exogenous active Pcsk9 protein. Co-administration of Pcsk9 [3 ng ml^{-1}, based upon concentrations measured in the supernatants of untreated AML12 (*Figure 4D*)] and SRT3025 to AML12 hepatocytes attenuated the drug-dependent increase in Ldlr protein expression (*Figure 5A*). These data indicate that the internalization and degradation process of Ldlr is not defective and that limited extracellular availability of Pcsk9 contributes to the increase in Ldlr protein expression.

Fluorescence analysis revealed an increase in labelled LDL uptake upon 10 μM SRT3025 treatment compared with vehicle controls (*Figure 5B*), suggesting a *functional* relevance of the SRT3025-dependent increase in Ldlr expression. Moreover, co-administration of exogenous active Pcsk9 also attenuated the drug-induced increase

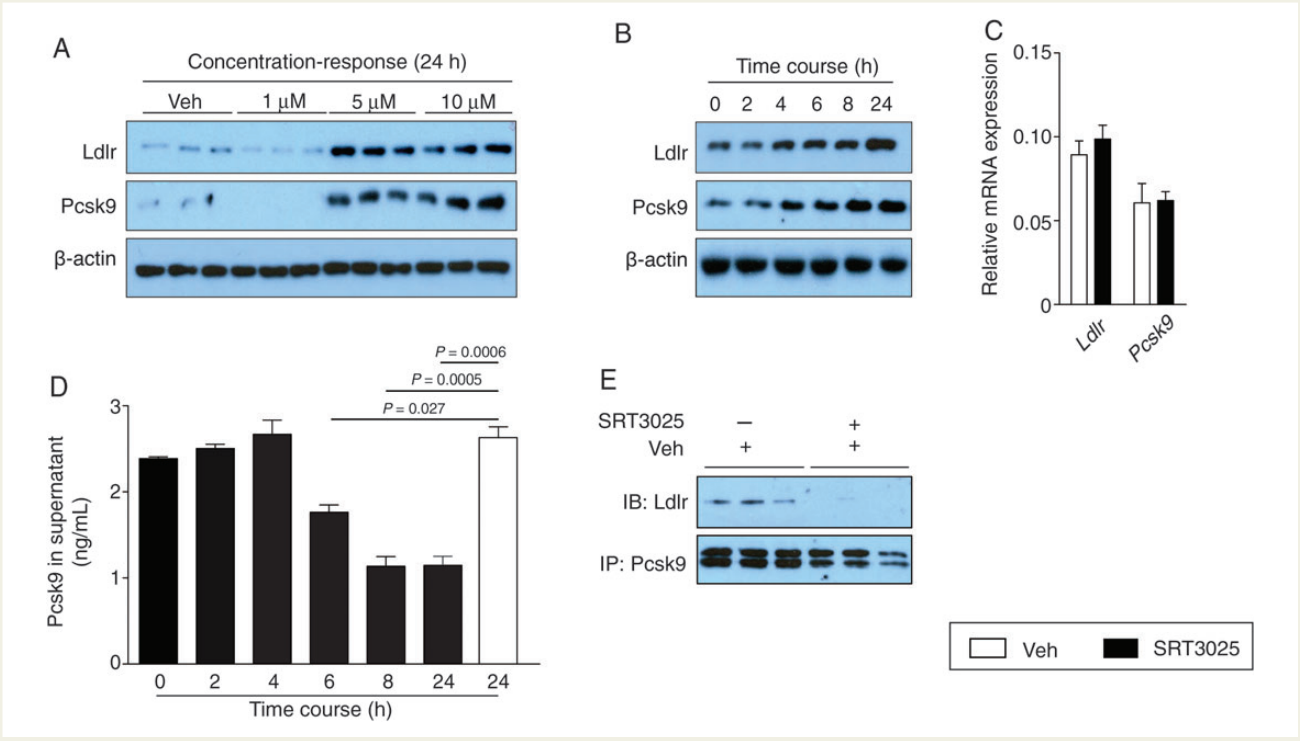

Figure 4 SRT3025 increases Ldlr expression in AML12 hepatocytes and decreases Pcsk9 in the supernatant. Western blots of Ldlr, Pcsk9, and β-actin in cultured AML12 cells (*A*) treated with SRT3025 at indicated concentrations for 24 h and (*B*) incubated with 10 μM SRT3025 for the times indicated. (C) Relative mRNA expression levels of *Ldlr* and *Pcsk9* in AML12 cells after incubation with 10 μM SRT3025 for 24 h. (*D*) Pcsk9 protein levels in the supernatant of AML12 cells incubated with 10 μM SRT3025 for the times indicated. (*E*) Pcsk9 immunoprecipiated from AML12 cells incubated with vehicle (Veh, DMSO) or 10 μM SRT3025, and blotted for Ldlr and Pcsk9. Pcsk9, proprotein convertase subtilisin/kexin type 9.

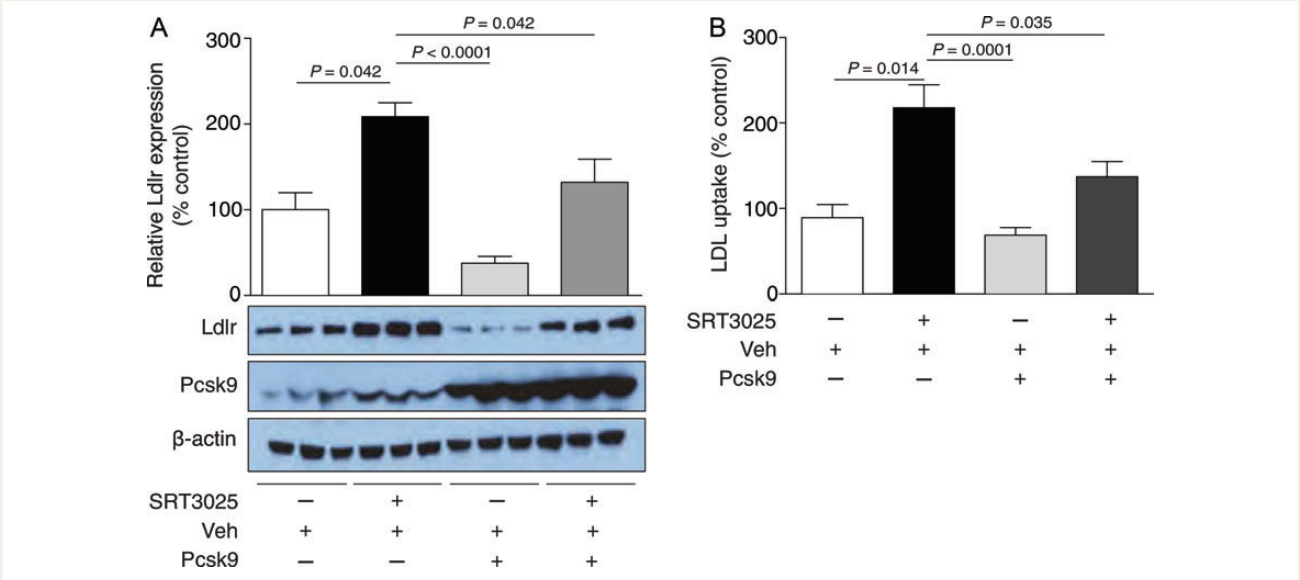

Figure 5 Exogenous Pcsk9 prevents SRT3025-induced increase in Ldlr expression and activity in AML12 hepatocytes. (*A*) Western blot and corresponding quantifications of AML12 cells treated with vehicle (Veh, DMSO) or 10 μM SRT3025 and incubated with or without Pcsk9 active protein (3 ng ml^{-1}) for 1 h. (*B*) BODIPY-labelled LDL uptake in AML12 cells incubated for 24 h with 10 μM SRT3025 or Veh and incubated with or without Pcsk9 active protein (3 ng ml^{-1}) for 1 h. Fluorescence intensity and western blot quantifications are given as percentage of Veh control. BODIPY, 4,4-difluoro-3a,4a-diaza-s-indacene; AU, arbitrary units; Pcsk9, proprotein convertase subtilisin/kexin type 9.

in LDL uptake (*Figure 5B*), consistent with the observed attenuation of Ldlr protein expression (*Figure 5A*).

These data indicate that SRT3025 decreases hepatic Pcsk9 release, impairs Pcsk9 binding to hepatic Ldlr, and thereby prevents hepatic Ldlr degradation. These events result in increased hepatic Ldlr expression and enhanced LDL-cholesterol plasma clearance.

Increased Ldlr protein expression in AML12 hepatocytes is mediated by Sirt1

To assess whether this increase in Ldlr protein expression is Sirt1-dependent, *Sirt1* knockdown in AML12 cells was performed in the presence of 10 μM SRT3025. In contrast to control scramble siRNA-treated cells, cells transfected with *Sirt1* siRNA did not show an increase in Ldlr expression upon SRT3025 addition (*Figure 6A*). Conversely, genetic overexpression of *Sirt1* increased Ldlr protein expression in AML12 cells compared with control transfection (*Figure 6B*). Thus, Sirt1 is required for SRT3025-induced increase in Ldlr protein expression.

Genetic deletion of Ldlr abolishes atheroprotective effects of SRT3025 in vivo

Given the critical role of hepatic Ldlr in the clearance of plasma cholesterol and our observation of increased hepatic Ldlr expression in SRT3025-treated *Apoe*$^{-/-}$ mice, we investigated whether Ldlr accounts for the atheroprotective effects of SRT3025. For that purpose, similar experiments were performed in *Ldlr*$^{-/-}$ mice. Plaque analyses of thoraco-abdominal aortae *en face* and cross-sections of aortic roots revealed no difference in the extent of atherosclerosis between drug- and placebo-treated mice (*Figure 7A* and *B*). Furthermore, the number of plaque-resident macrophages (Cd68) and Vcam-1 expression in aortic roots did not differ between the groups (*Figure 7C* and *D*).

Plasma lipids showed an increase in total and VLDL-cholesterol in SRT3025-treated *Ldlr*$^{-/-}$ mice compared with placebo-treated controls (*Figure 7E* and *F*), whereas plasma LDL-cholesterol, HDL-cholesterol, and triglycerides were unaffected by drug treatment (*Figure 7F* and Supplementary material online, *Figure S2A*). As in *Apoe*$^{-/-}$ mice, SRT3025 prevented an increase in weight gain and epididymal white adipose tissue without affecting food intake (Supplementary material online, *Figure S2B–D*), and lowered plasma levels of pro-inflammatory cytokines Mcp-1 and Il-6 (*Figure 7G*). Compared with *Apoe*$^{-/-}$ mice, baseline plasma Pcsk9 levels in *Ldlr*$^{-/-}$ mice were about 20-fold higher (*Figure 7G*). SRT3025 induced a minimal but significant increase in plasma Pcsk9 levels in *Ldlr*$^{-/-}$ mice (*Figure 7G*). Pharmacological Sirt1 activation decreased the expression of *Pparα*, but did not affect *Abcg5*, *Abcg8*, *Abca1*, *Pcsk9*, *Pparγ*, *Srebp1*, *Srebp2*, *Mcp-1* and *Il-6* (Supplementary material online, *Figure S2E*).

Discussion

We demonstrate that pharmacological Sirt1 activation using SRT3025 attenuated Pcsk9 secretion from murine hepatocytes *in vitro* and lowered plasma levels of Pcsk9 in atherosclerosis-prone *Apoe*$^{-/-}$ mice *in vivo*. As a consequence, hepatocyte Ldlr expression and activity were increased leading to a decrease in plasma LDL-cholesterol and atherosclerotic plaques in *Apoe*$^{-/-}$ mice. None of these variables were changed in *Ldlr*$^{-/-}$ mice despite a similar decrease in systemic inflammation. Thus, our findings identify reduced Pcsk9 secretion and increased Ldlr expression as novel downstream effects of Sirt1 activity and highlight the potential of pharmacological Sirt1 activation as a novel anti-atherosclerotic strategy.

SRT3025 was found to activate wild-type Sirt1 protein but failed to activate the E230K mutant, an activation-resistant Sirt1 protein

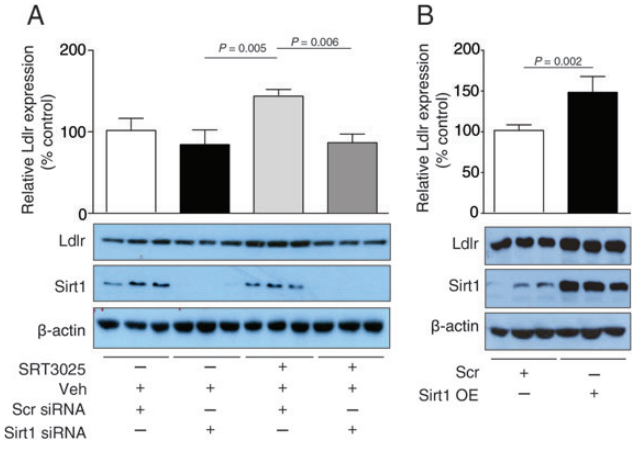

Figure 6 Sirt1 knockdown reduces and Sirt1 overexpression enhances SRT3025-induced increase in Ldlr expression in AML12 hepatocytes. (*A*) Western blots of Ldlr, Sirt1, and β-actin with corresponding quantifications of AML12 cell lysates following transfection with Sirt1 siRNA or scrambled siRNA for 24 h and incubated with 10 μM SRT3025 or vehicle (Veh, DMSO) for additional 24 h. (*B*) Western blots of Ldlr, Sirt1, and β-actin with corresponding quantifications of AML12 cell lysates following Sirt1 overexpression plasmid or scramble control for 24 h and incubated with 10 μM SRT3025 or Veh for additional 24 h. Western blot quantifications are given as a percentage of Veh control. Scr, scramble plasmid; Sirt1 OE, Sirt1 overexpression plasmid.

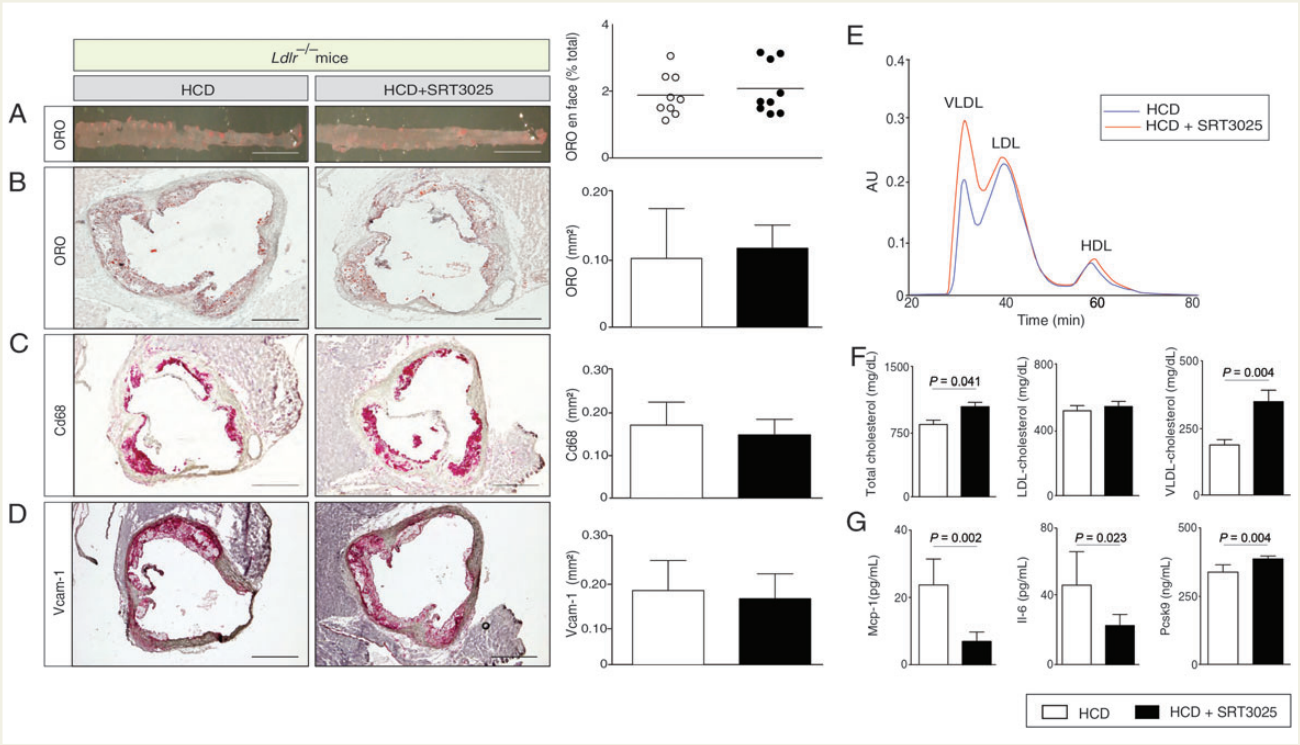

Figure 7 Genetic deletion of Ldlr abolishes anti-atherosclerotic effects of SRT3025 *in vivo*. Eight-week-old *Ldlr*$^{-/-}$ mice were fed a high-cholesterol diet (1.25% w/w) supplemented with the Sirt1 activator SRT3025 ($n = 9$) or placebo ($n = 9$) for 12 weeks and fasted for 12 h before blood was drawn and aortae were explanted. Representative micrographs (left) and quantifications (right) of thoraco-abdominal aortae *en face* (*A*) and of aortic root cross sections (*B*–*D*) stained with oil-red O (ORO) or immunohistochemically for macrophages (CD68) or Vcam-1; (*E*) cholesterol distribution in the different lipoprotein subfractions separated by gel filtration chromatography; (*F*) plasma total cholesterol, LDL-, and VLDL-cholesterol concentrations; (*G*) plasma levels of Mcp-1, Il-6, and Pcsk9. Scale bars in photomicrographs = 1 mm (*A*) and 500 μm (*B*–*D*). HCD, high-cholesterol diet; AU, arbitrary units; LDL, low-density lipoprotein; ORO, oil-red O; VLDL, very low-density lipoprotein; Vcam-1, vascular cell adhesion molecule 1; Mcp-1, monocyte chemoattractant protein 1; Il-6, interleukin 6; Pcsk9, proprotein convertase subtilisin/kexin type 9.

(due to a mutation at position 230). Thus, similar to previously described Sirt1 activators,[15] SRT3025 acts by allosteric binding to Sirt1 at the lysine site 230, which is located at its catalytic core. Analyses of the acetylation status of the Sirt1 target Foxo1 in skeletal muscle and p65 in the liver show that SRT3025 increases deacetylation of Sirt1 target proteins *in vivo*. Furthermore, the increase in Ldlr protein expression in AML12 cells *in vitro* in response to stimulation with the Sirt1 activator was time- and concentration- dependent and could be attenuated by knockdown of *Sirt1*, while genetic *Sirt1* overexpression mimicked the effects of SRT3025. These data indicate that the SRT3025-associated changes in Ldlr expression are Sirt1-dependent.

Endothelial-specific overexpression of *Sirt1* in *Apoe*$^{-/-}$ mice was reported to protect against atherosclerosis by increasing endothelial eNOS activity.[2] Furthermore, using partial *Sirt1* deletion in *Apoe*$^{-/-}$ mice, we have shown that Sirt1-induced inhibition of NFκB impairs endothelial expression of the macrophage scavenger receptor Lox-1, and thus attenuates foam cell formation.[3] Although these findings address beneficial effects of Sirt1 in a particular setting of atherosclerosis, they provide limited insight into the effects of Sirt1 on cholesterol metabolism. Interestingly, hepatic deletion of *Sirt1* in C57BL/6 mice fed a high-cholesterol diet was associated with a mild increase in plasma LDL-cholesterol,[7] suggesting that hepatic Sirt1 regulates plasma LDL-cholesterol. In line with these findings, *Apoe*$^{-/-}$ mice treated with SRT3025 showed an increase in hepatic Ldlr expression accompanied by a significant reduction in plasma LDL-cholesterol levels. Moreover, the extent of aortic atherosclerosis and the plasma levels of LDL-cholesterol were unchanged in *Ldlr*$^{-/-}$ mice, highlighting the importance of Ldlr for the atheroprotective and lipid-lowering effects of Sirt1. Of note, we observed a similar reduction in plasma levels of pro-inflammatory cytokines in both *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, whereas a reduction in plaque formation was only observed in *Apoe*$^{-/-}$ mice with endogenous Ldlr. Therefore, our data suggest that not the reduction in inflammation, which still occurs in the absence of Ldlr, but the decrease in plasma LDL-cholesterol accounted for reducing atherosclerosis in *Apoe*$^{-/-}$ mice treated with SRT3025. Moreover, since we observed a reduction in weight gain in both *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice despite unaltered food intake, the atherosclerotic phenotype is also independent of changes in body weight. Taken together, we demonstrate that the increased expression of hepatic Ldlr is essential for the cholesterol-lowering effects of SRT3025.

In line with our findings, clinical studies with another Sirt1 activator have shown a reduction in plasma total cholesterol and

LDL-cholesterol.[8,9] The presently observed reduction in VLDL-cholesterol in mice has yet to be assessed in patients. Furthermore, constitutive Sirt1 overexpression in *Ldlr*$^{-/-}$ mice was associated with increased atherosclerosis.[5] Pharmacological Sirt1 activation using an oral Sirt1 activator and constitutive genetic *Sirt1* overexpression are likely to differ in efficiency and may exert differential effects on genes involved in lipid metabolism. Moreover, a difference in NAD^+ availability between both models may affect Sirt1 activity.[16] It remains to be seen whether constitutive *Sirt1* overexpression is associated with enhanced hepatic expression of Ldlr in *Apoe*$^{-/-}$ mice.

Ldlr plays a critical role in the regulation of plasma LDL-cholesterol levels,[10,11] a key determinant of atherogenesis.[17] Mice overexpressing hepatic Ldlr have reduced plasma LDL-cholesterol levels and are protected from plaque formation, whereas *Ldlr*-deficient mice exhibit increased levels of plasma LDL- and VLDL-cholesterol and are prone to atherosclerosis when exposed to a high-cholesterol diet.[4,18] We show that Ldlr protein expression is increased despite no change in *Ldlr* mRNA, indicating a post-translational mechanism. Indeed, degradation of Ldlr protein in liver cells is regulated via secreted Pcsk9, a serine protease that predominantly originates from the liver.[14,19] By binding to the EGF-A domain of Ldlr, Pcsk9 targets this receptor for lysosomal degradation rather than for recycling to the cell surface.[20,21] Ldlr was reported to be the main route of Pcsk9 clearance.[22] In agreement with this, we found markedly increased plasma Pcsk9 levels in mice lacking *Ldlr*.

In our study, both SRT3025 treatment and *Sirt1* overexpression increased Ldlr protein expression in AML12 cells, while reducing the concentration of Pcsk9 in the supernatant and lowering the amount of Pcsk9 bound to Ldlr, despite no change in Pcsk9 transcription. Notably, exogenous addition of active Pcsk9 to AML12 cells treated with SRT3025 decreased Ldlr protein expression and reduced LDL uptake by the cells. These data indicate that Ldlr internalization and degradation are not disrupted by SRT3025 treatment and can be re-induced upon exogenous addition of Pcsk9. Moreover, these data demonstrate that it is the limited extracellular availability of Pcsk9 that contributes to the increase in Ldlr protein expression, suggesting that plasma Pcsk9 levels are an important determinant of hepatic Ldlr protein surface expression in our model. The mechanisms by which Sirt1 affects Pcsk9 secretion remain to be investigated. Yet, the specific investigation of Pcsk9-dependent alterations on atherosclerosis may be hampered by reciprocal changes in Ldlr.[22]

Conclusions and perspectives

Our study describes a novel mechanism that causally links Sirt1 to the metabolism of LDL-cholesterol *in vitro* and *in vivo*. We demonstrate that Sirt1 activation increases hepatic Ldlr protein expression through a reduction in Pcsk9-mediated Ldlr degradation leading to decreased plaque formation.

Of note, statin therapy not only increases hepatic Ldlr, but also plasma Pcsk9 activity, thus limiting its effect on LDL-cholesterol.[23] Antibody-induced inhibition of Pcsk9 binding to Ldlr is currently investigated as a very promising therapy to reduce plasma LDL-cholesterol and atherosclerosis in patients.[24,25] Our study demonstrates a Sirt1-dependent alternative route to target Pcsk9 activity in a mouse model. Thus, a Sirt1 activator combined with a statin may confer the advantage of lowering plasma Pcsk9 activity. Future studies are needed to translate the effects of pharmacological Sirt1 activation to patients.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: D.A.S. is a consultant to GlaxoSmithKline (GSK), J.L.E. an employee at GSK. V.S. and J.L.E. were employees of Sirtris Pharmaceuticals, a GSK company. B.S. is a member of the *Institut Universitaire de France*. T.F.L. received research grant by Sirtis (now GSK) to the institution. J.E. is an employee of GSK. V.S. reports personal fees from GlaxoSmithKline Inc., during the conduct of the study; personal fees from GlaxoSmithKline Inc, outside the submitted work; D.S. reports personal fees from GlaxoSmithKline, during the conduct of the study; personal fees from Ovascience, and MetroBiotech, grants from Caudalie and NovoNordisk, outside the submitted work; In addition, D.S. is also an inventor on patents on molecules to activate sirtuins and increase survival of mammalian cells with royalties paid by GlaxoSmithKline.

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The Sirt1 activator SRT3025 provides atheroprotection in *Apoe*$^{-/-}$ mice by reducing hepatic Pcsk9 secretion and enhancing Ldlr expression

SUPPLEMENTAL ONLINE MATERIALS (SOM)

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Supplemental Methods

Cholesterol profiles and lipoprotein fractions

Cholesterol and triglyceride plasma concentrations were measured using colorimetric and enzymatic methods with ready to use kits (BioMérieux). To assess the distribution of cholesterol and triglycerides over the different lipoprotein fractions, lipoproteins were separated by size exclusion chromatography followed by online determination of lipids.[1]

Transfections

Sirt1 gene silencing in AML12 cells was accomplished by transfecting mouse *Sirt1* siRNA (Dharmacon) using Lipofectamine (Invitrogen); Scramble siRNA (Microsynth) was used for negative control. *Sirt1* overexpression was accomplished by transfection of an overexpression vector and a scramble empty vector as control (provided by Johan Auwerx) using the Jet Pei transfection protocol.

LDL uptake

AML12 hepatocytes were incubated with 5 μg/ml of BODIPY-labelled human LDL (L-3483, Invitrogen) in 6-well plates in serum-free medium for 1 h at 37 °C. After two washings with PBS, uptake of BODIPY-labelled LDL was determined by analysis of fluorescence using FacsCanto flow cytometer (BD Bioscience, Heidelberg, Germany), and the percentage of positive platelets calculated with FacsDiva software.

ELISA of plasma cytokines and Pcsk9

Monocyte chemoattractant protein 1 (Mcp-1) and interleukin 6 (Il-6) plasma levels were measured using an adipokine panel kit (Millipore); Pcsk9 was determined by ELISA (Circulex, MBL).

RNA and protein analyses

Total RNA was extracted using Trizol (Sigma-Aldrich) and reverse transcribed with random hexamers. cDNA obtained was quantified by SYBR-green qPCR using gene-specific primers. Results were normalized to Rps29 and Rps18. Total protein from tissues and cells was prepared as described and analysed using RIPA lysis buffer and standard protocols.[2] Protein was immunostained using specific antibodies targeted to Sirt1 (Cell signalling), Ldlr (Novus Biologicals), Pcsk9 (Abcam) or ß-actin, and detected by horseradish peroxidase chemiluminescence.

Immunoprecipitation

Foxo1 and p65 acetylation was assessed by immunoprecipitating Foxo1 and p65 from 1 mg of total proteins from liver, followed by immunoblotting against acetylated lysine (Abcam), anti-acetyl-Foxo1 (Santa Cruz) or anti-acetylK310-p65 (Abcam), respectively. Membranes were stripped and reblotted to assess loading with anti-Foxo1 (Santa Cruz) and anti-p65 (Abcam).

Immunohistochemistry

Serial cryosections from the aortic sinus were obtained from frozen tissues fixed in OCT. Cryosections were fixed in acetone, blocked in 2% normal mouse serum, and stained with oil-red O (ORO), anti-Cd68 (Abcam) and Vcam-1 (Abcam) antibodies. For *en face* analysis of atherosclerotic lesion area thoraco-abdominal aortae were fixed with 4% paraformaldehyde and plaques were stained with ORO and analysed as described.[3]

Sirt1 activity assay

Activation of Sirt1 proteins (wild-type and a Sirt1 activation-resistant mutant (E230K)) was measured using the O-Acetyl ADP ribose mass spectrometry assay (OAcADPR assay) as described. [4]

Supplemental Table 1: Plasma and tissue levels of SRT3025 in *Apoe*$^{-/-}$ mice

Tissue	HCD	HCD+SRT3025	Units
Plasma	nd	8.87 ± 2.33	µmol /L
Aortae	nd	2.67 ± 3.40	µmol /g
Liver	nd	8.26 ± 5.29	µmol /g

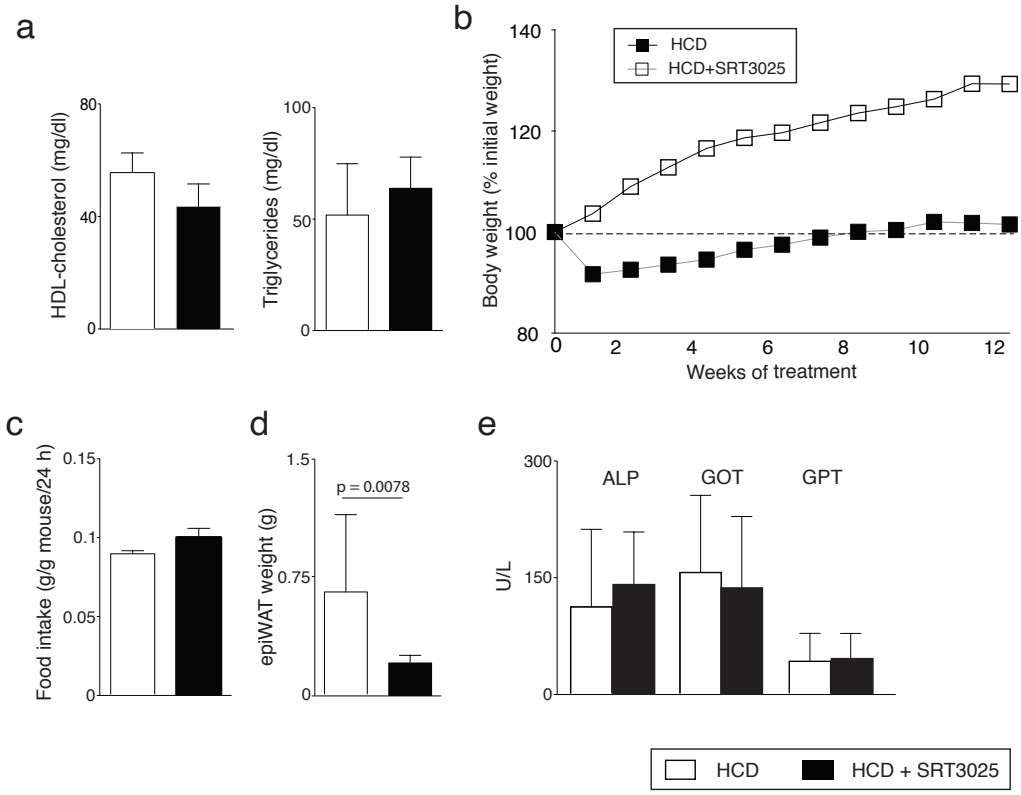
Plasma and tissue levels of SRT3025. Eight week-old *Apoe*$^{-/-}$ mice were fed a high-cholesterol diet (1.25% w/w) supplemented with the Sirt1 activator SRT3025 (n=9) or placebo (n=9) for 12 weeks. After this treatment period, mice were sacrificed (after overnight fasting), EDTA blood was taken and tissues were harvested. Levels of drug were measured by LCMS. nd, not detectable.

Supplemental Table 2

Primers

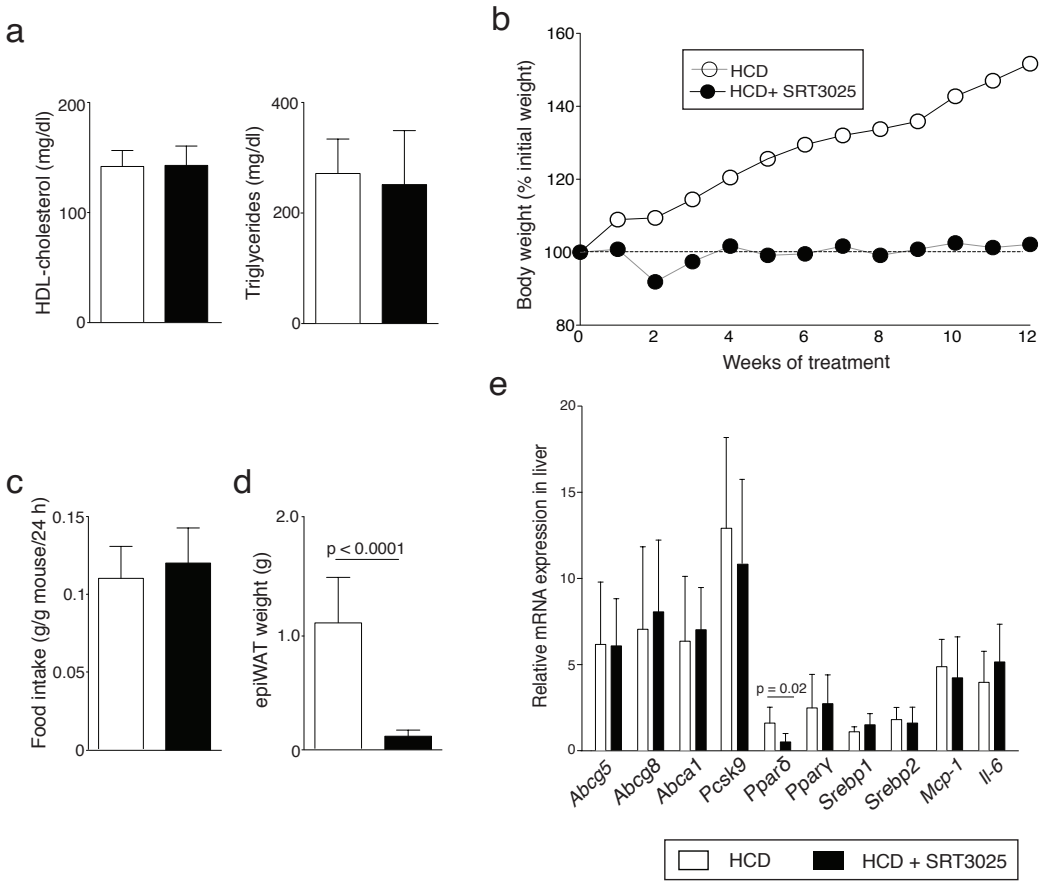
Gene	Forward Primer	Reverse Primer
Abcg5	*AGGGCCTCACATCAACAGAG*	*GCTGACGCTGTAGGACACAT*
Abcg8	*TCCGAGGAGAACAAGCTGTC*	*CACTGGTCATGGCTGAGAAA*
Abca1	*GCTGCAGGAATCCAGAGAAT*	*CATGCACAAGGTCCTGAGAA*
Ldlr	*AGTGGCCCCGAATCATTGAC*	*CTAACTAAACACCAGACAGAGGC*
Pcsk9	*GAGACCCAGAGGCTACAGATT*	*AATGTACTCCACATGGGGCAA*
Pparα	*AGAGCCCCATCTGTCCTCTC*	*ACTGGTAGTCTGCAAAACCAAA*
Pparγ	*TCGCTGATGCACTGCCTATG*	*GAGAGGTCCACAGAGCTGATT*
Lxr	*CTGATTCTGCAACGGAGTTGT*	*GACGAAGCTCTGTCGGCTC*
Rxr	*ATGGACACCAAACATTTCCTGC*	*CCAGTGGAGAGCCGATTCC*
Srebp1	*TGGTTGTTGATGAGCTGGAG*	*GGCTCTGGAACAGACACTGG*
Srebp2	*GCAGCAACGGGACCATTCT*	*CCCCATGACTAAGTCCTTCAACT*
Tnf-α	*CCCTCACACTCAGATCATCTTCT*	*GCTACGACGTGGGCTACAG*
Mcp-1	*TCAGCTGCCTGCAAAGACCAGA*	*ACGGTGTGGTGGCCCCTTCAT*
Il-6	*TAGTCCTTCCTACCCCAATTTCC*	*TTGGTCCTTAGCCACTCCTTC*
Rps18	AGTTCCAGCACATTTTGCGAG	TCATCCTCCGTGAGTTCTCCA
Rps29	GTCTGATCCGCAAATACGGG	AGCCTATGTCCTTCGCGTACT

Supplemental Figures



Suppl Figure 1. SRT3025 mimics a caloric restriction phenotype in *Apoe*$^{-/-}$ mice.

Eight week-old *Apoe*$^{-/-}$ mice were fed a high-cholesterol diet (1.25% w/w) supplemented with the Sirt1 activator SRT3025 (n=9) or placebo (n=9) for 12 weeks. Plasma HDL-cholesterol and triglycerides **(a)**; body weight course of *Apoe*$^{-/-}$ mice fed a HCD without (control) or with SRT3025 (S1A) and normalized to initial body weight before diet **(b)**; average daily food intake **(c)** and weight of epididymal white adipose tissue (epiWAT) **(d)**; hepatic enzymes: ALP, GOT and GPT **(e)**. HDL, high-density lipoprotein; HCD, high-cholesterol diet; ALP, alkaline phosphatase; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase.



Suppl Figure 2. SRT3025 mimics a caloric restriction phenotype in *Ldlr*$^{-/-}$ mice.

Eight week-old *Ldlr*$^{-/-}$ mice were fed a high-cholesterol diet (1.25% w/w) supplemented with the Sirt1 activator SRT3025 (n=9) or placebo (n=9) for 12 weeks. Plasma HDL-cholesterol and triglycerides **(a)**; body weight course of *Ldlr*$^{-/-}$ mice fed a HCD without (control) or with SRT3025 (S1A) and normalized to initial body weight before diet **(b)**; average daily food intake **(c)** and weight of epididymal white adipose tissue (epiWAT) **(d)**; hepatic gene expression of *Ldlr*$^{-/-}$ control mice and *Ldlr*$^{-/-}$ mice treated with SRT3025 for 12 weeks **(e).** HDL, high-density lipoprotein; HCD, high-cholesterol diet.

Supplemental References

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6. Perspectives

"A man is as old as his arteries"

—Thomas Sydenham, 1624–1689

6.1 SIRT1 activator SRT3025, atherosclerosis and dyslipidaemia

Our studies in mice with SRT3025 demonstrate that STACs can reduce atherosclerosis by stimulating SIRT1 activity and lowering plasma PCSK9 levels. Although the study of sirtuins and lipid metabolism is still in its infancy, increasing evidence shows that SIRT1 activation improves plasma lipid profile in mice and humans, thus creating an opportunity to treat dyslipidaemia.

Our findings show SRT3025 to be atheroprotective in ApoE$^{-/-}$ mice by reducing plasma LDL-cholesterol, highlighting the potential of SIRT1 activators for prevention or therapy in human cardiovascular disease. In addition, SRT3025 was found to reduce hepatic steatosis in diet-induced obese mice through a suppression of lipogenesis and an increase in fatty acid oxidation (Abstract, Qing Nie), further underlining the therapeutic potential of STACs. However, despite the fact that SRT3025 showed promising atheroprotective effects in mice, pharmacokinetic and safety studies of SRT3025 in humans showed a dose-dependent QTc prolongation (Jonathan Haddad, 2013). Thus, SRT3025 itself could not be pursued in human studies but provides evidence that SIRT1 activation would be an interesting target

against atherosclerosis. Clinical trials of another SIRT1 activator – SRT2104 administered to elderly volunteers and healthy cigarette smokers also showed reductions of plasma total cholesterol and LDL-cholesterol levels. Consistent with this, we found that SIRT1 activation by SRT3025 reduces plasma LDL-cholesterol in ApoE$^{-/-}$ mice by reducing the secretion of PCSK9 in the plasma and increasing hepatic LDL-R expression. An increase in LDL-R causes an increase in LDL-cholesterol uptake leading to reduction in plasma LDL-cholesterol. Although the effect of SRT3025 was shown to be SIRT1-dependent, further investigation is required to understand the mechanism for the reduction in PCSK9 secretion. Furthermore, the plasma PCSK9 levels of patients receiving SIRT1 activators still need to be investigated.

6.2 Role of sirtuins in the regulation of PCSK9

Although these findings support the notion that SIRT1 activation reduces PCSK9 secretion, direct interaction of SIRT1 and PCSK9 has yet to be investigated. It is appealing to postulate that SIRT1 may directly deacetylate PCSK9. If SIRT1, indeed, deacetylates PCSK9, it would be informative to assess the difference in acetylation status of PCSK9 in diseased patients as a surrogate not only for SIRT1 activity but also for PCSK9 activity. Recently, it was shown that PCSK9 expression could also be regulated by SIRT6.[87] Such findings confirm that sirtuins indeed play an important role in PCSK9-mediated LDL-cholesterol regulation, which occurs both at a transcriptional and post-translational level. Since knockdown of SIRT1 blocks SIRT6 induction in hepatocytes, it can be postulated that absence of SIRT1 may cause a

reduction in PCSK9 regulation transcriptionally, rather than post-translationally. Further studies need to be done to assess whether other sirtuins regulate PCSK9 expression and to determine whether there is any cross talk between SIRT1 and SIRT6 in regulating plasma PCSK9 levels.

6.3 SIRT1, white adipose tissue and atherosclerosis

Subcutaneous white adipose tissue is known to be an active immune organ that stores excess calories from circulating lipids in adipocytes and secretes inflammatory adipokines and hormones. Adipocytes can secrete a number of pro-inflammatory adipokines, such as MCP-1 and IL-6.[88] SIRT1 is known to modulate proliferation and inflammation in adipose tissue by repressing adipocyte differentiation and macrophage infiltration.[89] In our experimental models of atherosclerosis, both $ApoE^{-/-}$ and $LDL\text{-}R^{-/-}$ mice treated with the SIRT1 activator, SRT3025, showed a reduction in circulating MCP-1 and IL-6. In line with this, the reduction in systemic cytokines corresponded to the reduced mass of subcutaneous white adipose tissue in both models. Thus it can be suggested that the reduction in subcutaneous WAT is associated with the reduction in inflammation. It would be interesting to assess if the reduction in inflammation and inflammatory cytokines are causatively related to SIRT1 activity on white adipose tissue mass and if this can be linked to obesity.

Obesity is known to cause insulin resistance, an enlargement of adipocytes and accumulation of lipids within adipocytes. SIRT1 is known to improve insulin sensitivity and protect against obesity by reducing adipocyte proliferation.[90] However, the relationship between obesity and cardiovascular

disease has been controversial at best. Results from the 'Seven Countries Studies' epidemiological study showed that there was little relationship between body weight and coronary artery disease.[91] It however showed a strong association between blood cholesterol levels and coronary artery disease. Furthermore, in a major autopsy study called 'The Geographic Pathology of Atherosclerosis' in which autopsies from Africans were compared with New Yorkers, the authors concluded that obesity had little relationship with atherosclerosis and that serum lipid content may be more important.[92] However, it was the 'Framingham heart study' that associated the risk of atherosclerosis to increasing degrees of obesity.[93] Although all epidemiological studies had shortcomings on the design of their studies, many critics of the Framingham heart study claimed that the risks were related to major risk factors like cholesterol, blood pressure and diabetes and not obesity itself. In line with this, in our study we found no change in atherosclerosis in LDL-$R^{-/-}$ mice, although body weight was decreased. In $ApoE^{-/-}$ mice treated with SRT3025, plasma LDL-cholesterol and total cholesterol were lower and were associated with to the reduced WAT mass and inflammatory cytokines phenotype; however, in LDL-$R^{-/-}$ mice treated with the same drug, plasma LDL-cholesterol and total cholesterol was not associated with the reduced levels of WAT mass and inflammatory cytokines. If obesity indeed were a direct risk factor of atherosclerosis in mice, the reduction of body weight and fat in LDL-$R^{-/-}$ mice would be atheroprotective, which was not the case. Our study further shows that atherosclerosis in mice was primarily due to altered levels of only one major risk factor – LDL-

cholesterol, and not body-weight, fat mass or inflammation in the mice. Further studies of atherosclerosis in an obese mouse model lacking LDL-R or ApoE would help prove that atherosclerosis may be prevented by only reducing LDL-cholesterol and is independent of obesity or inflammation.

Since atherosclerosis is a chronic inflammatory disease triggered by elevated levels of LDL-cholesterol, a lot of research has focused on inflammation and inflammatory markers as prognostic biomarkers for the progression or risk of atherosclerosis. Our findings show that inflammation during atherosclerosis could be a secondary effect, independent of plasma LDL-cholesterol levels. Comparison of all inflammatory markers in the plasma of $ApoE^{-/-}$ mice and $LDL\text{-}R^{-/-}$ mice treated with SRT3025 by proteomics would reveal novel causative inflammatory biomarkers of atherosclerosis which could be used in prognosis of the disease. These inflammatory biomarkers would be more closely associated with plasma lipid content rather than systemic inflammation. Such a strategy would also provide biomarkers independent of those secreted by white adipose tissue and directly related to SIRT1 activity. Given the reduction in WAT in both models, the cytokines released from WAT such as IL-6 and MCP-1 may also prove to be of less importance in the search for biomarkers of atherosclerosis.

6.4 Alternative pathways of SIRT1 activation: increasing NAD^+ levels

The expression and activity of SIRT1 is dependent on the intracellular availability of its substrate NAD^+. Thus modulation of intracellular NAD^+ levels could be another method to increase SIRT1 activity and mimic its beneficial

effects. This can be achieved by two different strategies – increasing NAD^+ biosynthesis or decreasing NAD^+ consumption. During the biosynthesis of NAD^+ within cells, the enzyme nicotinamide phosphoribosyltransferase (NAMPT) catalyses the rate limiting step of converting nicotinamide to nicotinamide mononucleotide (NMN) and pyrophosphate.[94] NMN is then converted to NAD^+ by the isoenzyme nicotinamide mononucleotide adenylyltransferase.

The physiological role of NAD^+ biosynthesis is well understood, however its role in diseases is yet unclear. Extracellular NAMPT has been shown to behave as an insulin-mimetic and an inflammatory cytokine.[95, 96] Thus, pharmacological activation of NAMPT may not be an attractive strategy to increase NAD^+ levels and SIRT1 activity. Supplementation with NMN was shown to protect mice hearts from ischemia-reperfusion injury by increasing SIRT1 activity and upregulating NAMPT levels. These protective effects were lost in cardiac-specific SIRT1 knockout mice, thus providing evidence that exogenous supplementation with NMN may be used to increase NAD^+ levels and activate SIRT1. Currently few studies have addressed the modulation of NAD^+ levels by supplementation of tryptophan and nicotinic acid (NA). Tryptophan is the main precursor required for the *de novo* biosynthesis of NAD^+, while nicotinic acid is another NAD^+ precursor required by the Preiss-Handler pathway. Dietary supplementation of both precursors in animals are known to increase intracellular levels of the precursor and thus cellular NAD^+ levels.[97 98] Bioavailability of NAD^+ is critical for normal cellular functions, thus cells can also retrieve NAD^+ through the salvage pathway.

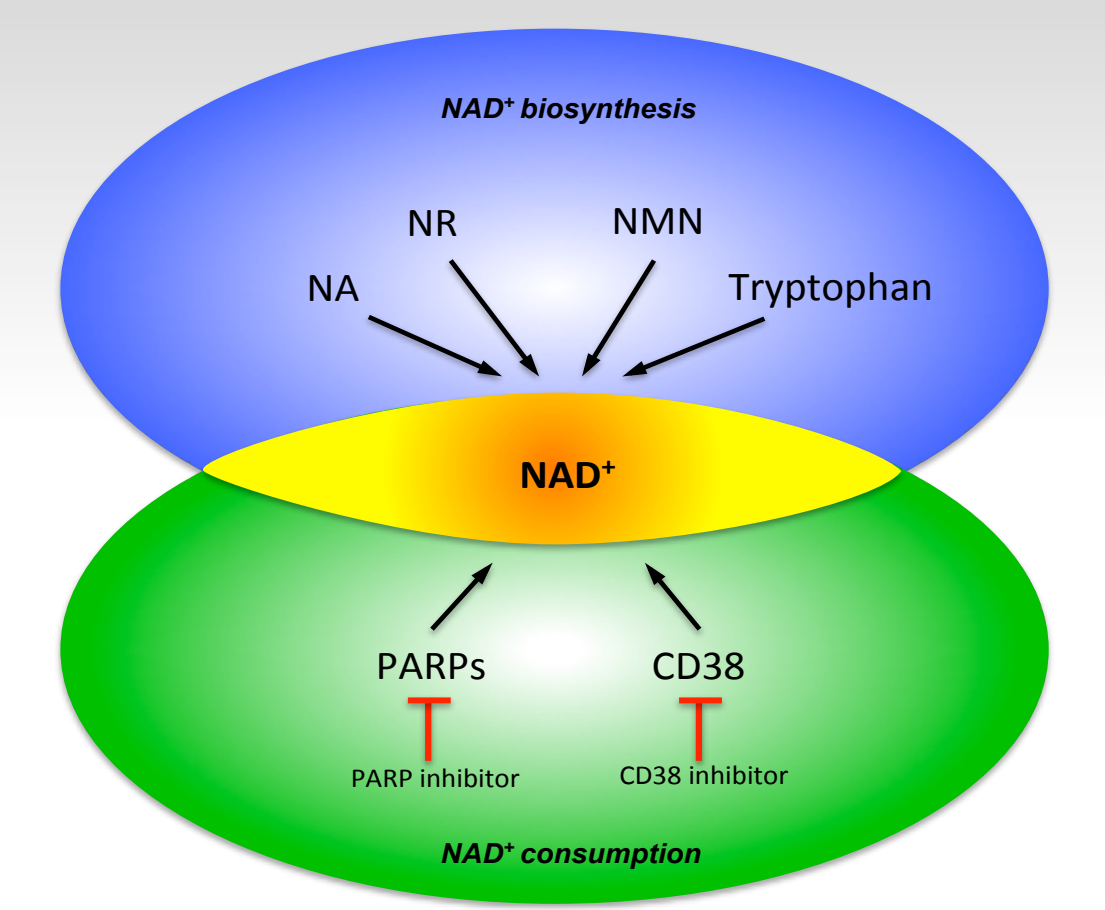

Figure 8. Modulation of NAD^+ biosynthesis and consumption. *Since activity of SIRT1 depends on the availability of NAD^+ in the cells, different strategies can be employed to increase cellular NAD^+. Intracellular NAD^+ biosynthesis can be increased by supplementation with NAD^+ precursors like nicotinic acid (NA), nicotinamide ribose (NR), nicotinamide mononucleotide (NMN) and tryptophan. The consumption of NAD^+ can be reduced by inhibition of NAD^+ consuming enzymes such as poly (ADP-ribose) polymerase (PARP) and CD38.*

The main precursors for the salvage pathway are nicotinic acid, nicotinamide (NAM) and nicotinamide riboside (NR). Since NA and NAM have comparative metabolism in mice, both can lead to higher levels of NAD^+ in

tissues, which may also be tissue-specific.[99] NAM seems to be a more stable precursor of NAD^+ in the liver, NA in the kidney and NR in neurons.[99-101] While the effects of NA and NAM have been studied through niacin, the function of NR has yet to be investigated. Niacin, a vitamin mainly composed of NA and NAM is used to treat hypercholesterolemia as it has beneficial effects on lipid metabolism. Niacin decreases VLDL-cholesterol and plasma LDL-cholesterol levels and increases plasma HDL-cholesterol levels. [102, 103] It is not yet known whether these beneficial effects are due to SIRT1 activation. However, similar to SIRT1, niacin increases HDL-cholesterol levels through an increase in ABCA1 expression. Since niacin has already been established as a lipid-lowering agent, the question remains whether more specific activators of NAD^+ biosynthesis cause further lowering of plasma cholesterol.

Another approach to increase SIRT1 activity through NAD^+ levels is by decreasing the consumption of NAD^+ within cells. Poly ADP-ribose polymerase (PARP) and CD38 are two enzymes that utilize NAD^+ as a substrate for their cellular processes. While PARP utilizes NAD^+ to build ADP-ribose polymers, CD38 uses NAD^+ to increase secondary messengers, mainly cADP-ribose.[104, 105] Loss of PARP-1 in $ApoE^{-/-}$ mice can protect against vascular dysfunction through an increase in eNOS activity.[106, 107] Currently PARP inhibitors are being investigated in a clinical setting for the treatment of cancer due to their role in DNA damage and apoptosis. Although inhibition of PARPs and CD38 has been shown to increase NAD^+ levels,[108, 109] further studies are required to assess if these inhibitors can provide beneficial effects in a clinical setting through SIRT1.

6.5 Future prospects

SIRT1 activation itself is a promising concept but the road from bench to bedside for drugs may be long and tedious. There are many obstacles and checkpoints for SIRT1 activators to pass before they can be used in the treatment of human diseases. Given the various target substrates of SIRT1, the differential deacetylation of SIRT1 substrates by STACs, their potential off-target effects and the various pathways involved in different diseases, it will be a challenge to establish the therapeutic potential of the currently approved SIRT1 activators.

Future studies on the mechanism and specificity of SIRT1 activation will support the discovery of novel drugs that could activate SIRT1 and exhibit target specificity. These premises may allow them to be tested for atheroprotection in patients.

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9. Credit points

Course	Points	Years
Blood pressure Practicals	0.5	2011
Hearing Practicals	0.5	2012
ECG Practicals	0.5	2012
Eye Practicals	0.5	2012
Project management for researchers	1	2012
Self-marketing Skills - Improve Your Interactional Performance	1	2012
Understand Intercultural Differences	1	2012
Respiratory physiology	1	2013
Flow cytometry course	1	2013
Applying statistical methods in Biosciences	1	2013
Scientific Writing	1	2014
Competency Awareness – the Foundation of a Confident Self-Presentation	1	2014
Pre-doctoral examination	2	2014

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E D U C A T I O N

2011-Current **University of Zurich, Zurich Cardiovascular Research laboratory / Center for Molecular Cardiology, Institute of Physiology and University Heart Center, Zurich**
PhD student in Integrative Molecular Medicine

2007-2008 **University of Oxford, Somerville College**
Master of Science in Pharmacology
- Passed with 69.2%
- Master dissertation project: Laboratory based under the guidance of Dr. Len Seymour
- Distinction in Essay examination Ranked 1st

2003-2007 **University of Mumbai, Mumbai Educational Trust, Institute of Pharmacy**
Bachelor of Pharmacy
- Passed first class

2001-2003 **University of Mumbai, Antonio Da Silva College**
Higher Secondary Certificate (HSC)
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W O R K E X P E R I E N C E

2009-2011 **Research Associate** **(**Center for Clinical and Molecular Physiology)

- Performed experiments and colony management of genetically engineered mice
- Investigated fumarate hydratase and hypoxia in kidney cancer models
- Developed Factor inhibiting hypoxia inducible factor (FIH) overexpressing cell lines

2008-June **Teaching assistant (*In vivo* skills)** (University of Oxford)

- *In vivo* surgery on guinea pigs
- Demonstrated the influence of drugs on respiration

2006-2007 **Production Chemist** (Glumex Pharmaceuticals, Mumbai, India)
- Maintained quality control checks on drug manufacturing protocols

E X P E R I M E N T A L T E C H N I Q U E S

- Tissue Culture
- Cloning
- Western blotting
- PCR, q-PCR
- Chromatin immunoprecipitation (CHIP)
- DNA genotyping
- *In vivo* surgery on mouse, guinea pig
- Cell death assays
- Virus handling
- Confocal microscopy
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2007	Presented a seminar on "Applications of heat shock proteins" during the 58th Indian Pharmaceutical Congress, Mumbai Vision 2020
2004	President of the Tsunami relief fund at Mumbai Educational Trust
2001	Gold medallist in State level Boxing Championships

A C T I V I T I E S

Memberships	Organizer of the University of Zurich Vision 2020 Lecture series
	Member of the Indian Pharmaceutical Association
	Member of Oxford Entrepreneur Society

A B S T R A C T P R E S E N T A T I O N S

2014	World Heart Congress 2014, Melbourne
2014	AGLA & Cardiovascular Biology Meeting 2014, Fribourg
2013	European Society of Cardiology (ESC) Congress, Amsterdam
2013	MOVD 2013: 11th International symposium on mechanisms of vasodilatation, Zurich
2013	Cell Symposia Immunometabolism: From mechanisms to therapy, Toronto
2012	Zurich Center for Integrative Human Physiology, Annual Symposium 2012, Zurich

P U B L I C A T I O N S

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